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IN VITRO EVALUATION OF SOIL *Bacillus* STRAINS ISOLATED FROM THE BUCIM COPPER MINE FOR BIOCONTROL AGAINST GRAPEVINE DOWNY MILDEW

Natalija Atanasova-Pancevska[1], Dzoko Kungulovski[1], Denica Angelovska[1], Lina Mirkovik[1], Martina Stojanoska[1], Ognen Boskovski[1], Sofija Kostandinovska[1*]
[1]Ss. Cyril and Methodius University in Skopje, Faculty of Natural Sciences and Mathematics – Skopje, Institute of Biology, Department of Microbiology and Microbial Biotechnology

**Corresponding author: s.kostandinovska@gmail.com*

Abstract

Plasmopara viticola, the disease that causes grapevine downy mildew, is a major and enduring concern for the grapevine industry globally. It's challenging to manage using chemical and agricultural methods. For the wine-growing industry, several countries, including North Macedonia, have reoriented their strategies in order to reduce chemical inputs, which have been shown to be toxic and to have a negative impact on the environment. Instead, they are replacing these chemicals with sustainable biocontrol regulations. *Bacillus* spp. is a well-known plant-protective bacteria with antifungal potential in biocontrol strategies. The aim of this study was to isolate and screen soil *Bacillus* strains from the Bucim Copper Mine in four seasons, with potential applications for biocontrol of this disease. The highest number (3.2 x 10^5 $CFUg^{-1}$) of *Bacillus* spp. was found in autumn, while the lowest number (2.8 x 10^2 $CFUg^{-1}$) was found in winter. Out of 18 isolates, 4 showed antifungal activity against *Plasmopara viticola*. The intracellular metabolites of the isolates B_{1-19}, B_{2-3}, B_{3-2}, B_{3-4} showed maximum inhibition of 20-32 mm, while only the extracellular extract of the isolate B_{1-19} showed maximum inhibition of 25 mm against *Plasmopara viticola*. The moisture content values ranged from 0.9-8.5 % and the pH value ranged from 7.11 – 7.58. The total organic matter values ranged from 4.47 to 4.99%. Due to the antifungal potential as biological control agents against grape downy mildew, the isolates are expected to enhance integrated pest management systems going forward and maybe reduce the quantity of chemical fungicides used in vineyards.

Key words: *soil microorganisms, antifungal activity, phytopathogenic fungi, bioprotection*

INTRODUCTION

Grapevine downy mildew, caused by the oomycete *Plasmopara viticola*, is a severe disease that affects vineyards globally (Koledenkova et al., 2022). This pathogen infects all green parts of the vine, especially during warm and humid periods, causing rapid and extensive damage. To mitigate losses, regular fungicide applications are essential, as they protect against potential harm and significant economic impacts, which can reach up to 75% in humid grape-growing regions. Using chemical fungicides is a straightforward strategy against fungal diseases. However, it presents two significant challenges. Firstly, there is the issue of environmental pollution. Secondly, there is the emergence of fungal phytopathogen populations that become resistant to these chemical fungicides. In Japan, grape growers face significant challenges in managing fungal diseases, particularly due to the high adaptability of *Plasmopara viticola*, a major phytopathogen (Nityagovsky et al., 2024). This pathogen has shown a strong tendency to develop resistance to chemical fungicides. Specifically, resistant genes against quinone outside inhibitor and carboxylic acid amide fungicides were identified in *Plasmopara viticola* populations in Japanese vineyards in 2015 (Aoki et al., 2015). In the Republic of North Macedonia, downy mildew control in grapevines is primarily managed through the use of synthetic fungicides. However, the repeated and extensive application of these fungicides has led to the development of pathogen-resistant strains, the presence of residues, and environmental pollution (Kuzmanovska et al., 2023). *Plasmopara viticola*, the cause of downy mildew, is a major fungal disease affecting grapevines in North Macedonia. The incubation period, from infection to symptom appearance, is key for predicting disease development. Temperature is the primary factor influencing the disease, with lower temperatures delaying the incubation. Early infections often go undetected, delaying chemical treatments. Timing for spraying should focus on the end of the incubation period and favourable weather conditions, rather than vine growth stages, as temperature plays a critical role in disease progression (Bojkov et al., 2022a). Restricting fungicide use is essential to reduce environmental impact and residues, but the adaptability of Botrytis cinerea has led to resistance, often requiring additional treatments. Forecasting models address this by analysing microclimatic factors, such as temperature and humidity, to predict infection risk. This approach enables targeted fungicide application before symptoms appear, reducing spore dispersal and minimizing spray frequency, promoting sustainable disease management (Bojkov et al., 2022b).

Biological control using biofungicides offers an alternative strategy to chemical fungicides for managing diseases. Biofungicides utilize biological control agents, primarily microorganisms isolated from natural sources (Otoguro & Suziki, 2018). A wide array of these microorganisms has been identified and studied as potential candidates for biofungicides (Aoki et al., 2020). *Bacillus* spp. are among the microorganisms which are recognized for their ability to function as biocontrol agents, offering preferred option for managing a range of plant diseases. These bacteria, characterized by their gram-positive nature, ability to form spores, rod-shaped morphology, and motility, thrive in various environmental settings, predominantly in soil. For instance, *Bacillus subtilis* QST-713, marketed under the product name Serenade®, is commercially available and widely used to manage grey mould in viticulture (Rotolo et al., 2018). The purpose of this study was to evaluate the potential of the soil microorganisms from the genus *Bacillus* to control the grapevine downy mildew caused by *Plasmopara viticola*.The soil *Bacillus* strains were isolated from the Bucim Copper Mine in four seasons and screened for their capacity to inhibit the growth of *Plasmopara viticola* using the agar well diffusion assay.

MATERIAL AND METHODS

Sampling procedure and soil geochemical parameters

The soil samples were collected from the Bucim Copper Mine with coordinates N41°39'10.068" E22°21'22.5792", at altitude of 616 m. The temperature at the time of collection in spring season was 21 °C, 35 °C for summer season, 25 °C for autumn and 4 °C for winter season. All the samples were transferred to the lab. Then, the moisture content, pH of the soil and the organic carbon content were determined as described before (Atanasova-Pancevska et al., 2023).

Isolation and identification of *Bacillus* spp.

To enhance the growth of spore - forming bacteria, the soil samples were diluted in 0.9% saline solution and heated to 70 °C for 30 minutes, as part of the isolation process. Subsequently, serial dilutions of the sample were plated on nutrient agar (NA) plates and incubated overnight at 37 °C. Qualitative analyses were conducted based on the morphological characteristics of *Bacillus* spp.

Preparation of *Bacillus* spp. and *Plasmopara viticola*

A strain of the phytopathogenic fungi *Plasmopara viticola* was provided by the Department of Microbiology and Microbial Biotechnology at the Faculty of Natural Sciences and Mathematics, Skopje, North Macedonia. The fungus was grown on potato dextrose agar (PDA) at 25 °C for 10 days and stored on PDA slants at 4 °C. *Bacillus* spp. were incubated at 30 °C on nutrient agar (NA) plates for 24 hours. Next, the isolates were centrifuged for 20 minutes at 4000 rpm. After centrifugation, the resulting mixture was filtered through 0.22 μm biofilters to acquire sterile supernatant (Sha et al., 2020).

Antagonistic activity of *Bacillus* spp. by *in vitro* assay

Antagonism assay was conducted on PDA and NA Petri dishes using the well diffusion method with slight modifications (Atanasova-Pancevska, 2023). Each plate had four wells with an 8 mm diameter. Each well received 40 µL of the culture supernatant and the collected bacterial cells, the precipitates of each isolate. A diphenyl console fungicide (50 µL / 100 ml) was used as a positive control. The evaluation of antagonistic activity was conducted by measuring the diameter of inhibition zones (mm) surrounding wells where no observable growth of the tested microorganism was detected, following 72 to 96 hours of incubation at 25°C.

RESULTS AND DISCUSSION

Sampling procedure and soil geochemical parameters

The soil geochemical parameters for each collected sample are shown in Table 1. The pH value and organic carbon content was approximately the same for each sample. As the pH of the soil varies between 7.11 and 7.58, the corresponding humus content ranges between 4.47% and 4.99%.

Table 1. Soil geochemical parameters for the collected soil samples from Bucim Copper Mine in four different seasons.

Season	Moisture content (%)	Soil ph	Humus (%)
Spring	1.65%	7.31	4.97%
Summer	0.95%	7.48	4.47%
Winter	1.81%	7.11	4.90%
Autumn	8.5%	7.58	4.99%

These results indicate variations in moisture content, soil pH, and humus content across different seasons. Spring (1.65%) and winter (1.81%) show lower moisture content compared to autumn (8.5%), which has significantly higher moisture levels. There's a slight variation in pH, with autumn having the highest pH. Humus content also shows slight variability, with autumn having the highest percentage. The results show a correlation between soil pH and humus content.

Another study indicates a qualitative relationship where pH influences Soil Organic Carbon (SOC) dynamics, including humus. Specifically, lower pH (acidic conditions) is associated with reduced SOC and humus content, which underscores the importance of soil pH management in sustaining soil organic matter levels (Voltr et al., 2021). A different study found a weak negative correlation between Total Organic Carbon (TOC) and pH at the European continental scale, indicating that higher TOC (humus) content tends to be associated with lower pH values in agricultural soils, but this relationship shows spatial variability influenced by regional factors like climate, soil type, and agricultural practices (Xu & Zhang, 2021).

Based on the obtained results from this study, generally, there appears to be a trend where higher pH values correspond to slightly higher humus content, though the differences are relatively small in this dataset. This suggests a potential relationship between soil pH and humus accumulation, where soil pH may influence the decomposition and accumulation rates of organic matter, thus affecting humus content. Based on the obtained data, there appears to be no clear correlation between moisture content and soil pH, and also between moisture and humus content. There seems to be a slight positive correlation between soil pH and humus content. Higher soil pH values (such as in autumn) tend to correspond to slightly higher humus content.

Isolation and identification of *Bacillus* spp.

The bacteria isolates were isolated and streaked on NA plates. A total of 18 isolates were identified as *Bacillus* spp. based on their macroscopic and microscopic characteristics (Figure 1).

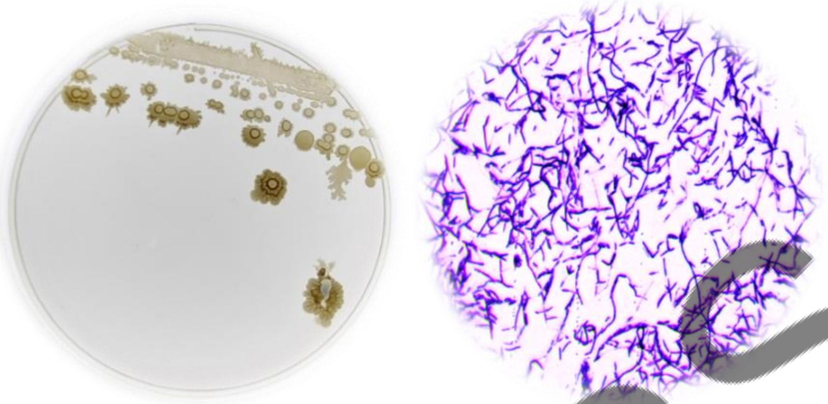

Figure 1. Macroscopic (left) and microscopic (right) characteristics of the strain B_{3-4} isolated from soil from Bucim Copper Mine in season autumn.

Antagonistic activity of *Bacillus* spp. by *in vitro* assay

Results from the well diffusion assay showed that out of 18 isolates, 4 showed antifungal activity against *Plasmopara viticola*. The intracellular metabolites of the isolates B_{1-19}, B_{2-3}, B_{3-2}, B_{3-4} showed maximum inhibition of 20-32 mm, while only the extracellular extract of the isolate B_{1-19} showed maximum inhibition of 25 mm against *Plasmopara viticola* (Figure 2).

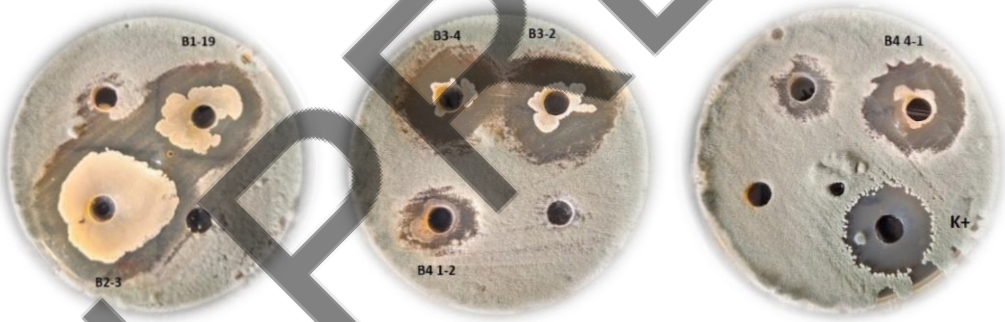

Figure 2. Well diffusion assay with *Plasmopara viticola*, with inhibition zones (20 – 32 mm) after 96 hours of incubation.

The present study demonstrates that the selected four *Bacillus* isolates decrease the incidence of downy mildew disease caused by *Plasmopara viticola* in vineyards. It is important to note that in the present study the isolates from the season winter did not show any antifungal activity. The maximum inhibition was observed from the isolate B_{3-4} isolated from season autumn. This can be correlated with the enumeration of *Bacillus* spp. from the soil in different seasons, since the highest number (3.2×10^5 $CFUg^{-1}$) of *Bacillus* spp. was found in autumn, while the lowest number (2.8×10^2 $CFUg^{-1}$) was found in winter. *Bacillus subtilis* is widely studied and used in agriculture for its production of biological control products due to its ability to produce various antibiotic compounds that combat fungi. It forms resilient endospores under harsh conditions, ensuring prolonged survival. Importantly, *Bacillus subtilis* and its by-products are considered safe, making it a preferred choice for global industrial fungicides (Furuya et al., 2011). The study by Zhang et al. (2017) demonstrated that the endophytic bacterial strain *Bacillus subtilis* GLB191 isolated from grapevine leaves effectively prevents *Plasmopara viticola* infection in susceptible grape cultivars Muscat Hamburg and Cabernet Sauvignon. The protective activity was observed in both leaf disk assays and field trials using GLB191 culture supernatant containing bacterial cells and metabolites, particularly cyclic lipopeptides (CLPs) such as surfactin and fengycin. The GLB191 supernatant induced callose production and upregulated defence gene expression in grapevine, suggesting it acts as a defence inducer against *Plasmopara viticola*. Mutant studies revealed that both surfactin and fengycin are crucial for GLB191's biocontrol activity, with each CLP contributing to both direct antifungal effects and plant defence stimulation against downy mildew.

Grapes face significant losses from diseases like grey mould, ripe rot, and downy mildew, prompting interest in biological control agents like *Bacillus strains* such as KOF112. *Bacillus*-based agents offer potential against a broad spectrum of fungi, reducing reliance on chemical fungicides and potentially curbing resistance development. For instance, *Bacillus velezensis* FZB42 produces antimicrobial peptides and polyketides, influencing plant defence systems. KOF112, isolated from grapevine shoot xylem, induces plant defences against fungal pathogens through enzymes like chitinase and ß-1,3-glucanase (Hamaoka et al., 2021).

Biological control agents, such as bacteria and fungi, are increasingly favoured over chemical pesticides due to their safety and sustainability. They effectively suppress plant diseases through mechanisms like competition, antibiosis, predation, and parasitism. Unlike chemical alternatives, these agents do not accumulate in food chains or pose significant risks to human health and the environment. By reducing reliance on toxic chemicals, biological control agents promote healthier agricultural practices. Strategies such as environmental manipulation, combining beneficial organisms, enhancing biocontrol mechanisms, adjusting formulations, and integrating with other methods can boost the effectiveness of biocontrol products. These approaches hold potential for sustainable agriculture by promoting plant growth (Sindhu et al., 2016). Choosing biocontrol agents effective across varied conditions like soil type, moisture, temperature, and competition is crucial. Publication of negative data is essential for identifying and addressing weaknesses in biopesticides, such as inconsistent field performance or economic viability, to drive future improvements (Lahlali et al., 2022).

CONCLUDING REMARKS

In conclusion, the study conducted at Bucim Copper Mine underscored seasonal variations in soil parameters such as moisture content, pH, and humus levels, with autumn exhibiting notably higher moisture and pH compared to other seasons. The correlation between soil pH and humus content suggests pH plays a role in organic matter accumulation. *Bacillus* spp. isolates from the soil displayed promising antifungal activity against *Plasmopara viticola*, highlighting their potential as biological control agents in vineyards. *Bacillus subtilis*, known for its production of effective biological control products emerges as a viable solution for combating grapevine diseases sustainably. Overall, biological control agents offer safe alternatives to chemical pesticides, promoting healthier agricultural practices and environmental sustainability. Future research should focus on optimizing these biocontrol strategies to enhance their efficacy across diverse agricultural conditions.

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IN VITRO ЕВАЛУАЦИЈА НА ПОЧВЕНИ ВИДОВИ ОД РОДОТ *Bacillus* ИЗОЛИРАНИ ОД РУДНИКОТ БУЧИМ ЗА БИОКОНТРОЛА НА БОЛЕСТА ПЛАМЕНИЦА КАЈ ВИНОВАТА ЛОЗА

Наталија Атанасова-Панчевска[1], Џоко Кунгуловски[1], Деница Ангеловска[1], Лина Мирковиќ[1], Мартина Стојаноска[1], Огнен Бошковски[1], Софија Костандиновска[1*]

[1]Универзитет „Св. Кирил и Методиј“ – Скопје, Природно – математички факултет, Институт за биологија, Катедра за микробиологија и микробна биотехнологија

**Контакт автор:* s.kostandinovska@gmail.com

Резиме

Plasmopara viticola, предизвикувачот на болеста на пламеница кај виновата лоза, претставува сериозен и долготраен проблем за виновата индустрија ширум светот. Управувањето со оваа болест преку хемиски и земјоделски методи претставува предизвик. Во повеќе земји, вклучувајќи ја и Северна Македонија, се превземаат стратегии за намалување на употребата на хемиските препарати, кои имаат негативно влијание на животната средина и се токсични. Замена на овие хемикалии со одржливи биоконтролни регулативи претставува веќепостоечка пракса. Бактериите од родот *Bacillus* претставува потенцијални кандидати за биоконтрола и заштита на растенијата. Целта на оваа судија беше изолација и скринирање на соеви од родот *Bacillus* од рудникот Бучим во четири временски сезони, со потенцијална примена за биоконтрола на болеста. Највисокиот број (3.2 x 10^5 $CFUg^{-1}$) на *Bacillus* соеви беше евидентиран во есен, додека најнискиот број 3.2 x 10^5 $CFUg^{-1}$) беше евидентиран во зима. Од 18^{те} изолати, 4 покажаа антифунгална активност кон *Plasmopara viticola*. Интрацелуларните метаболити на изолатите B_{1-19}, B_{2-3}, B_{3-2}, B_{3-4} покажаа максимална инхибиција од 25 mm, додека само екстрацеулалрните екстракти од изолатите B_{1-19} покажаа максимална инхибиција од 25 mm кон *Plasmopara viticola*. Содржината на влага на примероците се движеше во ранг од 0.9 – 8.5 % и pH вредностите во ранг од 7.11 – 7.58. Вкупната органска материја се движеше во ранг од 4.47 до 4.99 %. Балагодарение на антифунгалниот потенцијал како биолошки агенс за контрола на пламеница на виновата лоза, овие изолати имаат потенцијал да придонесат за развој на интегрирани системи за менаџмент на штетници во иднина и соодветно, да го намалат квантитетот на хемиски фунгициди кои се употребуваат во рамките на лозарството.

Клучни зборови: *почвени микроорганизми, антифунгална активност, фитопатогени фунги, заштита.*