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CONTENT

INTRODUCTION

Climate change and environmental protection are two critical challenges of the 21st century. Data from measurements of climate parameters show that the climate has been changing since the earliest records, even before the Industrial Revolution, due to natural processes. Today, when we talk about climate change, we refer to the changes that have been occurring since the beginning of the 20th century and they are the result of human activities.

Climate change is already having a visible effect, and numerous scientific papers and analyses predict and warn that it will occur even more intensively in the future. Climate change consequences are obvious, especially when it comes to its influence on the environment, biodiversity, plant and animal species, and finally human societies. Human life and societal well-being depend on natural resources, such as water, soil, flora and fauna. Although these resources are renewable, their unsustainable consumption and excessive contamination disrupt ecological balance, posing significant challenges to both nature and humanity.

Agricultural production is particularly vulnerable to climate change, because it operates as an "open-air factory". On the other hand, agriculture itself has a significant impact on climate change. Despite the technological progress in agriculture, utilization of improved crop varieties, application of biotechnology and enhanced irrigation management, climate and soil remain key to agricultural productivity. There is strong evidence for increasing uncertainty and variability of yields in agricultural production due to rising temperature fluctuations and the prevalence of various diseases and pests.

A growing number of studies suggest that agricultural land has the potential to mitigate the rise in atmospheric gases with the greenhouse effect. To address this, various global initiatives have been undertaken, with one promising solution being carbon sequestration in agricultural soils. The dynamics of carbon in the atmosphere and soil are complex and the amount of carbon that can be captured and stored in the soil depends on soil and crop type, implemented production practices, crop rotation, topography, and local climate. During photosynthesis, plants absorb carbon dioxide from the atmosphere and store it as carbon in their above- and below-ground biomass. At the end of their lifecycle, some of that carbon is released back into the atmosphere, while a portion remains stored in the soil for extended periods.

Many conventional agricultural practices result in higher carbon emission, whereas "carbon agriculture" practices aim to do the opposite. Carbon agriculture involves monitoring of carbon levels stored in the soil as result of production practices. These practices enhance carbon sequestration and reduce greenhouse gas emissions, leading to increased storage or sequestration of atmospheric carbon in living biomass, dead organic matter, and soil. Examples of these agricultural practices include soil conservation or minimal soil tillage, crop rotations involving a wider range of plant species, cover crops and proper management of post-harvest residues.

In this context, the Faculty of Agriculture, Goce Delcev University in Stip is participating in the project CARBONICA / Carbon Initiative for Climate Resilient Agriculture, funded by the European Union, together with three other partner institutions from the Republic of North Macedonia, six from Greece and four from Cyprus.

The main objective of the project is to strengthen the regional capacity for innovation through a multilateral approach and establish a long-term joint strategy for research and development in the field of agriculture with reduced greenhouse gas emissions, with a focus on initiating solutions to the challenge of transition to agriculture with reduced greenhouse gas emissions, through the incorporation of available knowledge, modern technologies and innovative policies. The project team is committed to achieving all project outcomes and jointly to contribute to building a sustainable future with carbon farming innovation.

December 2024

On behalf of JAPS Editorial Board,
Prof. Verica Ilieva, PhD
CARBONICA Project Manager for the Faculty of Agriculture,
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Original scientific paper

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

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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 (3.2×10^5 $CFUg^{-1}$) of *Bacillus* spp. was found in autumn, while the lowest number (2.8×10^2 $CFUg^{-1}$) was found in winter. Out of 18 isolates, 4 showed antifungal activity against *Plasmopara viticola*. The intracellular metabolites of the isolates $B_{1\text{-}19}$, $B_{2\text{-}3}$, $B_{3\text{-}2}$, $B_{3\text{-}4}$ showed maximum inhibition of 20-32 mm, while only the extracellular extract of the isolate $B_{1\text{-}19}$ 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.

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).

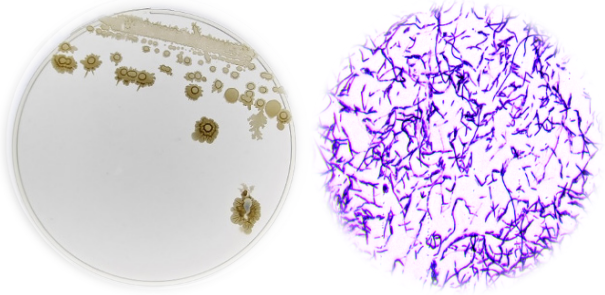

Figure 1. Macroscopic (left) and microscopic (right) characteristics of the strain $B_{3\text{-}4}$ isolated from soil from Bucim Copper Mine in season autumn.

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

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Резиме

Plasmopara viticola, предизвикувачот на болеста 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 преземаат 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* претставуваат потенцијални кандидати за биоконтрола и заштита на растенијата. Целта на оваа студија 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 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 екстрацеуларните 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 до 8.5 % и pH вредностите во ранг од 7.11 до 7.58. Вкупната органска материја се движеше во ранг од 4.47 до 4.99 %. Благодарение 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.*

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Professional paper

COMPARATIVE COST ANALYSIS OF SOIL CARBON DETERMINATION USING TOC ANALYZER *vs.* WALKLEY-BLACK METHOD

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Abstract

Soil carbon measurement is critical for understanding soil health, fertility, and the role of soils in global carbon cycling and climate change mitigation. Two widely used methods for determining soil organic carbon (SOC) are the Walkley-Black method and the use of Total Organic Carbon (TOC) analysers. Each method has unique strengths and limitations, making them suitable for different applications depending on accuracy, cost, and available resources. This study compares the costs associated with both techniques, including capital investment, consumables, labour, maintenance, and waste disposal. Moreover, the quality assurance comparative analyses have been applied as well, assuming the basic reference criteria, such as: precision, accuracy and uncertainty of the measurements. Data from multiple laboratories and sample scales are used to develop a comprehensive cost model. The analysis highlights the trade-offs between precision, scalability, and cost-effectiveness, offering insights for selecting the most suitable method for different applications.

Key words: *soil, carbon, TOC analyser, Walkley-Black method, cost-effective method.*

INTRODUCTION

Carbon in soil is crucial for maintaining soil health, fertility, and overall ecosystem stability. It primarily exists in the form of soil organic carbon (SOC), which originates from decomposed plant and animal materials (Bronick & Lal, 2005; Bienes et al., 2021). The importance of carbon in soil includes several key aspects, regarding: 1) soil structure and water retention, 2) nutrient supply, 3) microbial activity, 4) soil fertility and productivity and 5) carbon sequestration. Carbon improves soil structure by promoting the formation of aggregates, enhancing water-holding capacity, and reducing erosion (Qi et al., 2022). Organic matter rich in carbon provides a slow-release source of essential nutrients like nitrogen, phosphorus, and sulphur for plant growth (Ramesh et al., 2019; Bhattacharyya et al., 2022). Soil carbon supports diverse microbial communities that decompose organic matter, cycling nutrients back into forms that plants can absorb (Liang et al., 2017; Bhattacharyya et al., 2022). Higher carbon content is directly linked to increased soil fertility, which leads to better crop yields and sustainable agricultural practices (Merckx et al., 2001; Triberti et al., 2016; Coonan et al., 2020; Javed et al., 2022).

An accurate and cost-effective soil carbon analysis is crucial for sustainable agricultural management, climate change mitigation, and environmental monitoring. SOC is a key indicator of soil fertility, water retention, and structure, influencing crop productivity and ecosystem services (Paustian et al., 2019). Additionally, soil acts as a significant carbon sink, playing a vital role in regulating atmospheric carbon dioxide levels (Acharya et al., 2022).

Soil organic carbon measurements are essential for evaluating soil health, guiding fertilization strategies, and improving land productivity (Paustian et al., 2019). Accurate

data is necessary for quantifying carbon storage in soils, informing climate policies, and participating in carbon credit markets (Andries et al., 2021). Carbon sequestration monitoring involves measuring and tracking the capture and storage of atmospheric carbon dioxide (CO_2) in natural or artificial reservoirs to mitigate climate change. The process primarily occurs in soils, forests, and oceans, with soil carbon sequestration being a critical component for sustainable agriculture and land management. Accurate monitoring is essential for evaluating the effectiveness of sequestration practices and ensuring compliance with environmental policies and carbon credit systems. Tracking carbon sequestration contributes to understanding soil fertility, water retention, and ecosystem stability.

Moreover, reliable carbon measurements support research on global carbon cycles and the development of sustainable land use policies. Monitoring allows for accurate accounting of CO_2 removed from the atmosphere, supporting efforts to reduce global warming. Reliable data is necessary for trading carbon credits and verifying offsets in voluntary and regulated carbon markets. Data-driven insights help in formulating and adjusting policies aimed at reducing greenhouse gas emissions (Bibri et al., 2020; Luo et al., 2024). However, agriculture is an economic sector that requires large financial investments. Accordingly, not every farmer is financially able to monitor the carbon content. A general drawback is of course the availability of cost-effective but precise analyses. Cost-effective methods enable widespread soil testing, especially for large-scale agricultural operations and research projects (Heil et al., 2022). Lowering the cost of analysis allows better use of limited resources, enhancing monitoring frequency and geographic coverage. Affordable testing methods democratize access to soil health information for farmers, smallholder agriculturalists, and resource-limited regions (Bachmann et al., 2022).

Soil carbon is highly variable across locations and depths, primarily depended from the lithogenic and paedogenic environment (Lorenz et al., 2018; Lal et al., 2021). Soil carbon determination traditionally relies on dry combustion methods which require expensive equipment and high operational costs (Hammes et al., 2007; Chatterjee et al., 2009). Consequently, cost-effective alternatives are needed to facilitate broader soil carbon monitoring, especially in resource-limited settings. Techniques like the Walkley-Black method and Total Organic Carbon (TOC) analysers are frequently used to measure soil organic carbon (SOC) (Schumacher, 2002). In such research, it is necessary to make an assessment in balancing cost and accuracy of the selected methodology. Methods such as the Walkley-Black method and TOC analysers vary in cost, accuracy, and ease of use. Efficient selection of techniques based on project needs can optimize both budget and data reliability. Emerging technologies and hybrid approach further improve cost-efficiency without compromising accuracy.

Precise and accurate measurement of SOC is essential for sustainable land management and climate mitigation strategies. While the Walkley-Black method is a traditional chemical approach, the TOC analyser represents a modern, automated solution. Both methods have distinct cost structures influenced by equipment, consumables, labour, and maintenance.

The main goal of this paper is to introduce the comparative analysis for both soil carbon analysis methods, thus to: (1) evaluate the accuracy, based on the available published data (2) analyse cost and time efficiency, (3) recommend best practices for implementation. This paper aims to provide a detailed cost comparison, aiding decision-makers in selecting cost-efficient analytical approaches for various contexts.

MATERIAL AND METHODS

A separate evaluation of both methods was performed through a review of available data from manufacturers' technical specifications and available research articles for the application and validation of the methods.

Walkley-Black method

The Walkley-Black method, based on dichromate oxidation, is a standard chemical analysis technique, but its implementation cost can be significant depending on various factors. This study aims to develop a predictive cost model to estimate expenses associated with using this method, considering reagent consumption, labour, equipment, and waste management. It is a wet combustion method involving the oxidation of organic matter using potassium dichromate ($K_2Cr_2O_7$) in a sulfuric acid (H_2SO_4) medium. The methods principle is based on follow: the organic carbon in the soil is oxidized by potassium dichromate and concentrated sulfuric acid (Jha et al., 2014). The reaction generates heat, aiding the oxidation process. Excess dichromate that does not react with the organic matter is back-titrated with ferrous sulphate or ferrous ammonium sulphate to determine the amount of oxidant consumed. The analytical procedure includes: a known weight of soil (usually 0.5 to 1 g) is mixed with 1 N potassium dichromate solution. Then, the concentrated sulfuric acid is added, and the mixture is gently swirled to ensure complete reaction. The solution is left to cool. Excess dichromate is titrated with 0.5 N ferrous sulphate solution using an appropriate indicator (usually diphenylamine or orthophenanthroline). The organic carbon content is calculated based on the amount of dichromate reduced (Tóth et al., 2006; Stevens et al., 2008; Wight et al., 2016).

Cost components included in the methodology are given as follow:

- Chemicals: Potassium dichromate ($K_2Cr_2O_7$), sulfuric acid (H_2SO_4) and ferrous sulphate ($FeSO_4$) for titration;
- Labor: Time required for sample preparation, titration, and calculations;
- Equipment and consumables: Glassware (burettes, pipettes, flasks), balances and fume hoods;
- Waste Disposal: Costs associated with disposing of hazardous chromium-containing waste.

According to the set of variables for determining the real costs of applying the method, the following mathematical operation can be performed:

$$\boldsymbol{Total\ Cost} = C_{chemicals} + C_{labor} + C_{equipment} + C_{waste}$$

Where:

$$C_{chemicals} = \frac{Price\ per\ unit\ reagent \times Quantity\ used\ per\ sample}{Sample\ count}$$

$$C_{labor} = \frac{Hourly\ wage \times Processing\ time\ per\ sample}{Sample\ count}$$

$$C_{equipment} = depends\ on\ the\ depreciation\ over\ the\ equipment's\ lifespan$$

$$C_{waste} = depends\ on\ local\ disposal\ fees\ for\ hazardous\ materials$$

Soil Carbon Analysis with TOC Analyzer

Soil carbon analysis using a TOC analyser is commonly used analytical technique for monitoring the carbon cycle in agricultural land. This technique measures the amount of organic carbon in a soil sample, providing insights into the organic matter content (Qian & Mopper, 1996). The main key steps in TOC-based soil carbon analysis sample preparation, are given as follow: soil sample is submitted to air-dry process to remove the moisture. Sample is than ground and sieved in order to achieve a uniform particle size, typically less than 2 mm. Furthermore, for distinguishing between total carbon (TC) and inorganic carbon (IC), pretreatment with acid

(e.g., HCl) may be performed in order to remove inorganic carbon content (Bisutti et al., 2004; Sleutel et al., 2007).

The principle of analysis is based on combustion or chemical oxidation of the sample at high temperatures to release carbon dioxide. The released CO_2 is detected and quantified using infrared spectroscopy. TOC values can also be used to estimate soil organic matter (SOM), via a conversion factor (e.g., SOM ≈ TOC × 1.72) (Sleutel et al., 2007).

According to the set of variables for determining the real costs of applying the method, the following mathematical operation can be performed:

$$\boldsymbol{Total\ Cost} = \frac{C_{capital}}{L \times S} + C_{consumables} + C_{maintenance} + C_{labor} + C_{energy}$$

Where:

$C_{capital} = purchase\ cost\ of\ the\ TOC$

$L = lifespan\ of\ the\ equipment\ in\ years$

$S = numberof\ samples\ analyzed\ per\ year$

$C_{consumables} = cost\ of\ reagents\ and\ consumables\ per\ sample$

$C_{maintenance} = annual\ maintenance\ cost$

$C_{labor} = cost\ of\ operator\ time\ per\ sample$

$C_{energy} = energy\ cost\ per\ sample$

$C_{waste} = depends\ on\ local\ disposal\ fees\ for\ hazardous\ materials$

Data collection and model application

Data were collected from laboratory equipment vendors, consumable suppliers, and maintenance service providers. The model was applied to estimate costs for analysing sample sizes ranging from 10 to 100. The prediction variable for the increment of the sample number, especially for cases above 500. Data collection for the prices for both methods vary widely based on the type, features, and region. Prices evaluation for TOC analysers has been conducted covering available online data from the producers located in Europe, USA and China. The selection was based on three dominant producers, ranged the technology scale at three levels: prices for 1) Overview basic/entry-level models; 2) Mid-range models: main application for industrial and environmental monitoring applications; 3) High-end/Advanced models: mainly for research applications. The data collection for Walkley-Black method as traditional chemical analysis technique, involved evaluating factors: reagents, equipment and scalability at the one mid-range level, occupying producers from Europe, USA and China. The range of the applicability for Walkley-Black method was extracted as follow: 1) Low-throughput laboratories: with significant labour costs; 2) High-throughput laboratories: economies of scale in reagent and labour use.

RESULTS AND DISCUSSION

This paper aims to provide a detailed cost comparison, aiding decision-makers in selecting cost-efficient analytical approaches for various contexts. According to separate mathematical definitions for the total costs per method, critical variables that significantly affect the analytical processes are extracted, including satisfactory accuracy, precision and reproducibility in the application of the methods. Cost components for both methods are extracted into: a) Equipment

and capital costs: TOC analyser purchase vs. glassware and titration equipment for Walkley-Black; b) Consumables: oxygen gas and reagents for TOC; dichromate, sulfuric acid, and ferrous sulphate for Walkley-Black; c) Labor: time for sample preparation, analysis, and cleanup; d) Maintenance and repairs: service contracts for TOC vs. routine glassware replacement. e) Waste management: disposal of hazardous chromium waste for Walkley-Black method. Cost variables are defined for each method based on actual data from equipment manufacturers, chemical suppliers, and laboratory operations (Table 1). The representative sample was given for average of samples number of 100, minimum two operators per methods (for the labor cost) and average of 10 kg of waste.

According to the set of variables available for the both analytical methods, we are proposing a model for determining the real costs for comparative analysis of both methods. The following mathematical operation can be performed:

$$\boldsymbol{Total\ Cost_{method}} = C_{capital} + C_{consumables} + C_{maintenance} + C_{labor} + C_{waste}$$

Where:

$$C_{capital} = C_{equipment} + C_{instalation} + C_{software} + C_{trainng\ per\ hour}$$

$$C_{consumables} = C_{chemicals} + C_{initial\ materials} + C_{reference\ standards}$$

$$C_{maintenance} = C_{maintence\ per\ hour} + C_{replaceable\ materials} + C_{reference\ standards}$$

$$C_{labor} = C_{operating\ per\ hour} \times N_{operators} \times N_{working\ hours}$$

$$C_{waste} = C_{disposal\ fee\ per\ Kg} \times N_{operating\ days} \times 10$$

To obtain the final values for the individual variables, they were divided by 100 to obtain the price per sample. High variability in prices per sample is obtained for a representative total sample of more than 500 samples.

Table 1. Cost breakdown per sample (average costs values are given as Euro per sample).

Component	TOC analyser	Walkley-Black method
Capital equipment	5.00	0.50
Consumables	6.50	2.00
Labor	2.00	4.00
Maintenance	1.00	0.30
Waste disposal	0.10	0.70
Total	**14.6**	**7.50**

Cost data for Walkley-Black method were gathered from laboratory suppliers, labour rates, and waste disposal services. Chemical costs accounted for 25% of total expenses. Labor represented the largest cost burden of 55%, while equipment, maintenance and waste disposal shared the remaining 20%. A decrease in per-sample cost was observed with increasing batch sizes due to labour efficiency and bulk chemical pricing.

For TOC analyser method capital cost depreciation accounted for 35% of the total cost. Consumables represented the largest share at 45%, including oxygen gas and combustion tubes. Labor and maintenance each contributed approximately 20%. Costs decreased as the number of samples increased, showing economies of scale. Bulk purchasing of consumables and streamlined sample processing improved cost efficiency. The predictive model

enables laboratories and research institutions to estimate TOC analysis costs accurately. Strategies to reduce costs include maximizing equipment utilization, negotiating bulk pricing for consumables, and adhering to preventive maintenance schedules.

Cost behaviour across sample sizes

The costs associated with both methods generally include: 1) Fixed costs (unrelated to sample size): and 2) Variable costs (related to sample size). The ranged of the fixed cost for TOC analysers range from 20,000 to 80,000 Euros, Walkley-Black method range from 500 to 2000 Euros. Significant variation occurs for the variable cost (usually form 10-30% from the initial fixed cost). Costs per sample for TOC decrease with larger batch sizes due to capital cost amortization, whereas Walkley-Black remains relatively constant due to low capital investment (Table 1). Precision vs. cost TOC analysers offer superior precision and automation but require higher upfront investment and specialized maintenance. The Walkley-Black method is cost-effective for small-scale or low-budget projects but involves chemical hazards and manual labour. The Walkley-Black method generates hazardous waste requiring proper disposal, adding environmental costs not directly reflected in financial expenses. This comparative analysis demonstrates that TOC analysers are cost-effective for large-scale, high-precision applications, while the Walkley-Black method remains suitable for smaller, budget-limited projects. For low-throughput labs, the Walkley-Black method is cost-effective, but labour and safety concerns can add hidden costs. For high-throughput labs, a TOC analyser becomes more economical over time, especially when labour and reagent savings are considered.

Comparative analysis based on quality assurance (QA) of the methods

A comparative analysis based on the accuracy, precision and measurement uncertainty of TOC analysers and the Walkley-Black (WB) method evaluates their accuracy, reliability, and efficiency in measuring SOC content. The evaluation is conducted on the available data already published (Table 2). Regarding the precision and accuracy, TOC analyser provides highly precise and repeatable measurements by directly measuring carbon content using combustion and infrared detection. It minimizes human error and is suitable for low and high carbon concentrations, which results with lower mean values for standard deviation and variance (CV). Walkley-Black method as traditional chemical method involving increased time efficiency, export satisfactory precision, similar as TOC analyser. In addition to, WB method, demands time-consuming, requiring manual steps and careful titration, increasing the data inputs in the uncertainty indicator (Table 2).

Table 2. Data summary of comparative analysis of published improvements in QA for TOC analyser and WB method.

Sensitivity check	Improvement	TOC analyser	WB method	Data referenced from the past 2 decades:
Precision	MAE (%)	1.70	1.65	Weil et al., 2003; Bisutti, et al., 2004; Tóth et al., 2006; Sleutel et al., 2007, Stevens et al., 2008; Chatterjee et al., 2009; Petrokofsky et al., 2012; Da Silva Dias et al., 2013; Jha et al., 2014; Johns et al., 2015; Wight et al., 2016; Vitti et al., 2016; Davis et al., 2017; Nayak et al., 2019; Van Der Voort et al., 2023; Dupla et al., 2024.
Accuracy	SD (%)	0.45	0.52	
Uncertainty	CV (%)	26.5	32.5	

*MAE - Mean Absolute Error, SD - Standard Deviations, CV – Coefficient of Variation

Measurements for soil carbon using TOC analyser provides high accuracy, referred with values of analytical recovery in the range from 86 to 112%, as it uses direct measurement of organic carbon through combustion and detection of CO_2. The application of this methodology eliminates human error associated with manual titration. Results are typically more reproducible and reliable for a wide range of sample types. TOC Analyzer offers superior precision due to automated, standardized procedures. Moreover, repeatability is enhanced by advanced instrumentation with minimal human intervention.

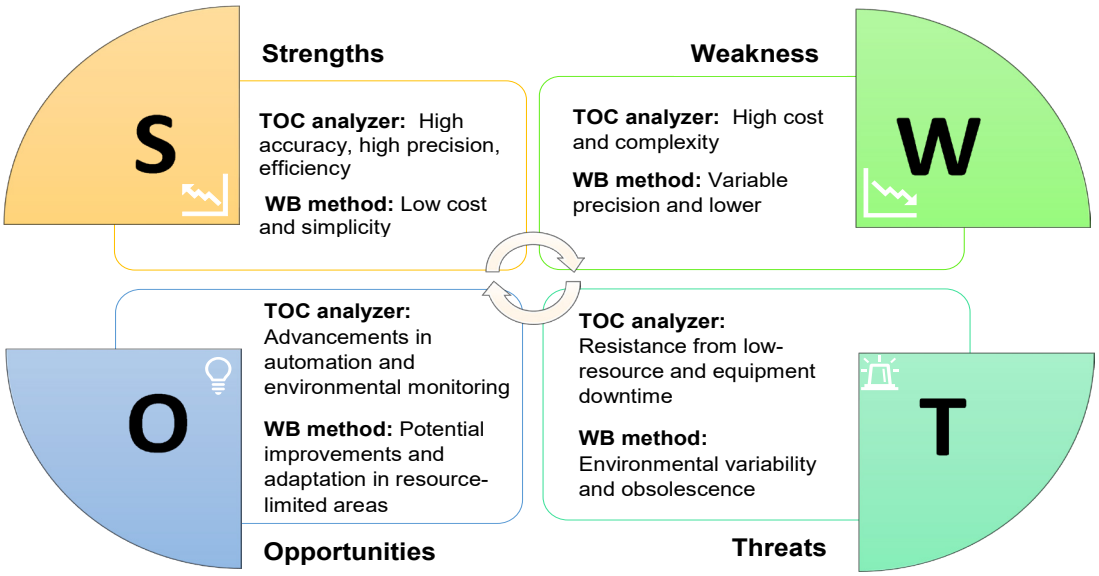
Walkley-Black method: Historically reliable but tends to underestimate TOC by 10-30% since it does not oxidize all organic carbon. Accuracy depends on the assumption of a fixed efficiency factor (often higher than 77%). The Walkley-Black method is often referenced for its susceptibility to analytical risks, including procedural errors and reagent quality concerns. Precision can vary based on the skill of the operator and consistency in handling reagents. Manual titration steps introduce higher variability of the reproducibility and repeatability of the measurements. The improvement of the reduced risk is conducted through the process of validation of the analytical procedure.

SWOT analysis

SWOT analysis summarizes key factors for the application of both methodologies (TOC and WB) for quantification of soil carbon content (Figure 1). Key indicators of both techniques were extracted using SWOT analysis. High accuracy of TOC analysers relays on direct combustion method, which ensures reliable carbon quantification. Automated process minimizes human error, resulting with incompatible high precision. Moreover, this methodology is suitable for high-throughput and diverse sample types. On the other side, the implementation of TOC analyser demands high initial investment and maintenance expenses. Furthermore, it requires technical expertise and specialized personnel. Thus, this methodology encounters resistance in low-resource settings due to its high cost. Routine monitoring programs often face equipment downtime, leading to an increased risk of analysis delays caused by maintenance requirements.

Walkley-Black method as traditional analytical procedure remains as the most economical and widely accessible methodology for soil carbon determination. This method requires only basic laboratory equipment and basic analytical skills. Moreover, is adaptable for routine analyses. Some laboratories refer to lower accuracy, due to the underestimates carbon content. The analytical recovery of approximately 70-80% results due to the partial oxidation of the organic compounds. However, this analytical risk can be decreased with validation process and implementation of control samples (reference materials or standard addition method). Mostly, results depend on operator skill and titration consistency. Thus, dominant opportunity for the WB application lies in its adaptation for use in resource-limited areas.

Figure 1. SWOT analysis TOC analyser vs. WB method.



CONCLUDING REMARKS

Investing in accurate, cost-effective soil carbon analysis supports sustainable land management, enhances climate resilience, and contributes to global efforts to combat climate change. Improving accessibility and affordability of reliable methods is key to maximizing environmental and economic benefits. Effective carbon sequestration monitoring is vital for climate action, sustainable agriculture, and economic incentives through carbon trading. Investing in reliable, cost-effective, and scalable monitoring systems will enhance global efforts to manage carbon and mitigate climate change.

Selection should be based on project scale, precision requirements, and available resources. The predictive cost model enables better financial planning for laboratories and research institutions. Cost per sample can be minimized by optimizing reagent use, training staff for efficiency, and investing in durable equipment. The proposed cost model provides a robust tool for predicting expenses associated with the Walkley-Black method. It supports strategic decision-making for large-scale soil carbon analysis initiatives. The TOC Analyzer is superior in both accuracy and precision, making it the preferred method for critical applications, while the Walkley-Black method remains valuable for cost-effective, routine SOC analysis with acceptable precision.

The TOC Analyzer leads in precision and accuracy but is limited by cost and complexity, while the Walkley-Black Method is cost-effective but less reliable, offering unique advantages for field application.

To summarise, maintaining adequate carbon levels in soil is vital for soil health, sustainable agriculture, and environmental protection. Soils act as major carbon sinks, storing carbon and mitigating climate change by reducing atmospheric carbon dioxide levels.

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КОМПАРАТИВНА АНАЛИЗА НА ТРОШОЦИ ЗА ОПРЕДЕЛУВАЊЕ НА ЈАГЛЕРОДОТ ВО ПОЧВАТА СО КОРИСТЕЊЕ НА TOC АНАЛИЗАТОР *vs.* WALKLEY-BLACK МЕТОДОТ

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Резиме

Мерењето на јаглеродот во почвата е критично за разбирање на плодноста на почвата и улогата на почвите во глобалниот циклус на јаглерод, како и намалување на ефектите на климатските промени. Два широко користени методи за одредување на органски јаглерод во почвата (SOC) се методот Walkley-Black и употребата на анализатори за одредување на вкупен органски јаглерод (TOC). Секој метод има уникатни предности и ограничувања, што ги прави погодни за различни апликации во зависност од точноста, цената и расположливите ресурси. Оваа студија ги споредува трошоците поврзани со двете техники, вклучувајќи капитални инвестиции, потрошен материјал, работна сила, одржување и отстранување на отпадот. Дополнително, применети се и компаративните анализи за обезбедување квалитет, при што се претпоставуваат основните референтни критериуми, како што се: прецизност, точност на мерењата, како и мерната неодреденост. Податоците од повеќе производители, дистрибутери и лаборатории се користени за да се развие сеопфатен модел на трошоци и квалитет на анализа. Извршената анализа на податоци ги екстрахира зависностите помеѓу прецизноста, приспособливоста и економичноста, нудејќи увид во изборот на најсоодветен метод за различни апликации и услови на примена.

Клучни зборови: *почва, јаглерод, TOC анализатор, Walkley-Black метод, економична метода.*

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Review paper

THE QUINONE OUTSIDE INHIBITOR FUNGICIDES, A PERSPECTIVE GROUP OF PLANT PROTECTION PRODUCTS

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Abstract

Strobilurins are a class of fungicides derived primarily from natural substances produced by wood-decaying fungi, particularly those in the genus Strobilurus. The discovery of strobilurins dates back to 1970s when researchers identified their unique fungicidal properties. Since then, various synthetic strobilurin analogues called Quinone outside inhibitors have been developed with a wide variety of applications. Due to their broad-spectrum activity and low toxicity to humans and animals, QoIs have become some of the most widely used fungicides worldwide. These fungicides are highly effective against a diverse range of fungal pathogens, including those responsible for diseases like powdery mildew, rusts, leaf spots, and blights. They are commonly used in crops such as cereals, field crops, fruits, nuts, vegetables, turfgrass, and ornamentals. Their systemic properties allow them to be absorbed into plant tissues and transported throughout the plant, providing protection to both treated areas and new growth. This enhances their efficacy in managing diseases. QoIs are compatible with many agricultural chemicals, such as insecticides and herbicides, making them ideal for integration into pest management programs. Their compatibility also allows for tank mixing, reducing the frequency of applications and saving time and resources for farmers. Additionally, some QoIs have been observed to promote plant growth under certain conditions. This review delves into various aspects of QoIs, including their mode of action, chemical and fungicidal properties, resistance, limitations and prospectives.

Key words: *strobilurins, QoI fungicides, plant protection, mode of action, fungicidal activity, resistance.*

INTRODUCTION

Strobilurins are versatile fungicides with a wide range of agricultural applications due to their broad-spectrum activity, systemic properties, and ability to enhance plant health. Their primary use is to manage fungal diseases across various crops targeting a broad range of pathogens which has made them indispensable in agriculture production. The discovery of strobilurins dates back to the late 1970s when Anke et al. (1977) isolated strobolurin A and strobilurin B from the basidiomycete fungus *Strobilurus tenacellus* (Pers. ex Fr.) Singer which grows on decaying cones of *Pyrus silverstris*. In fact, strobilurins are common secondary metabolites in Basidiomycetes. *B. lutea* is the only Ascomycete fungus known to produce metabolites from this chemical family (Iqbal et al., 2018). Although strobilurin A showed to be effective in "*in vitro*" it was not effective "*in vivo*" due to its low photochemical stability and high volatility. The discovery of the antimicrobial properties of strobilurins led to the synthesis of various analogues such as azoxystrobin which was introduced on the market for the first time in 1996 by Zeneca Agrochemicals (now part of Syngenta). This was the first synthetic strobilurin

fungicide, making a significant advancement in agricultural disease management due to its low toxicity and broad-spectrum activity, effective across a wide range of crops, such as fruits, vegetables, cereals, nuts, berries, turfgrasses ornamentals etc. Since it was discovered, azoxystrobin has had a profound impact on plant protection. By 2002, it was registered for use in 72 countries and on 84 different crops, achieving sales of $ 415 million in 1999, making it the top-selling fungicide worldwide (Bartlett et al., 2002). Moreover, its widespread adoption spurred the development of additional fungicides in this chemical group. The same year kresoxim methyl was developed and released on the market by BASF. Afterward, further developments in similar compounds quickly followed. Metominostrobin, was developed by Shionogi and released on the market in 1999. The same year Bayer lunched trifloxystrobin. In addition, other fungicidal compounds, chemically distinct from strobilurins but from the same QoI fungicide cross-resistance group were developed such as famoxadone by DuPont lunched in 1997 and fenamidone by Aventis in 2001, which broaden the spectrum of available options for disease control. In 2002, BASF lunched pyraclostrobin followed by picoxystrobin released by Syngenta the same year. More than a decade later in 2016, Sumitomo Chemical lunched on the market mandestrobin. Today, the class of QoI fungicides compares 21 substances belonging to ten chemically or biologically different group of compounds from which six are approved in EU, six are banned and one (metyltetraprole) has the status of pending for approval in EU (Tab.1). Six new QoI fungicides are in process of development by Chinese manufacturers. SRICI is working on coumoxystrobin, enoxastrobin, flufenoxystrobin, triclopyricarb, and fenaminstrobin, while Shenyang Sciencreat Chemicals is developing pyriminostrobin. However, it remains uncertain whether these companies intend to pursue global registration for these fungicides (Umetsu & Shirai, 2020).

The term 'strobilurins' originally referred to natural fungicidal compounds derived from fungi in the genus *Strobilurus*. Hence, the class also includes synthetic analogues with similar modes of action but different origins, the Fungicide Resistance Action Committee (FRAC) in the early 2000s when dealing with classification of fungicides based on their mode of action renamed the group into 'Quinone Outside Inhibitors' (QoI fungicides or QoIs) and classified them into the FRAC Code 11 to provide clarity and support resistance management strategies.

CHEMICAL PROPERTIES OF QoI FUNGICIDES

Fungi synthesize strobilurins from an essential α-amino acid called phenylalanine when synthesize biomolecules, such as proteins and secondary metabolites, through the shikimic acid pathway (Balba, 2007). The structural molecule of strobilurins contain the specified methyl (E)-3-methoxy-2-(5-phenylpenta-2,4-dienyl) acrylate moiety, attached to the α-position. Compounds with this structure are classified as β-methoxyacrylates (Tab. 1). The (E)-β-methoxyacrylate group is a critical part of their natural fungicidal activity (Bartlett et al., 2002). The α-position of the acrylate is connected to the rest of the strobilurin molecule, which can vary depending on the specific compound (Fig.1). Variations usually arise from substitutions on the aromatic ring at positions 3 and 4 (Fig. 1). However, these natural compounds degrade rapidly when exposed to light, which reduces their practical value and makes them unsuitable for effective plant disease control (Bartlett et al.,2002). This limitation of natural strobilurins has been overcome with development of photostable analogues with numerous practical applications (Iqbal et al., 2018; Kunova et al., 2021). Most modifications focused on altering the α-substitution of the (E)-β-methoxyacrylate group. A significant breakthrough and revolution in this field was achieved by replacing the core toxophoric (E)-β-methoxyacrylate group with a methoxyiminoacetate moiety, a modification which leads to the discovery of azoxystrobin (Fig. 1). Replacing the (E)-β-methoxyacrylate group with a (Z)-α-methoxyiminoacetate group lead to development of trifloxystrobin while a pyridine ring with trifluoromethyl substitution and methyl ester group attached to the methoxyiminoacetate group is a unique feature of fluoxastrobin that distinguishes it from other strobilurin fungicides (Fig 1). In kresoxim methyl a chlorine-substituted pyridine ring (6-chloro-3-pyridinyl) is attached to the methoxyiminoacetate

moiety (Fig. 1). A chlorinated pyridine ring (6-chloro-2-(chloromethyl)-4-pyridyl) and a methyl ester group are attached to the methoxyiminoacetate group in the molecule of pyraclostrobin (Fig. 1). One notable advancement replacing the (E)-β-methoxyacrylate group with 2-methoxyiminoacetamide, resulted in the creation of metominostrobin (Fig.1) (Bartlett et al., 2002). Mandestrobin also a methoxyacetamide based compound has a backbone derived from mandelic acid structure (Fig. 1) (Hirotomi et al., 2016).

Table 1. Classification and representatives of QoL fungicides (FRAC Code 11) according to the Fungicide Resistant Action Committee (FRAC, 2024c)

MOA	**C: respiration**									
target site and code	**C3 complex III: cytochrome bc1 (ubiquinol oxidase) at Qo site (cyt b gene)**									
Group name	**QoI-fungicides (Quinone outside Inhibitors)**									
FRAC CODE	**11**									**11A**
Chemical or biological group	methoxy-acrylates	methoxy-acetamide	methoxy-carbamates	oximino-acetates	oximino-acetamides	oxazolidine-diones	dihydro-dioxazines	imidazolinones	benzyl-carbamates	tetrazolinones
Status in EU	**approved non EU non EU non EU banned non EU**	**approved**	**approved non EU non EU**	**approved approved**	**banned non EU banned banned**	**banned**	**approved**	**banned**	**nonEU**	**pending**
Common name	**azoxystrobin coumoxystrobin enoxastrobin flufenoxystrobin picoxystrobin pyraoxystrobin**	**mandestrobin**	**pyraclostrobin pyrametostrobin triclopyricarb**	**kresoxim-methyl trifloxystrobin**	**dimoxystrobin fenaminstrobin metominostrobin orysastrobin**	**famoxadone**	**fluoxastrobin**	**fenamidone**	**pyribencarb**	**metyltetraprole**

MODE OF ACTION

A defining characteristic of QoI fungicides is their rapid mode of action. While most of the QoI fungicides are contact, some of them such as azoxystrobin, picoxystrobin and metominostrobin show systemic activity. Metominostrobin shows the highest ability to be absorbed into leaves while picoxystrobin and azoxystrobin showed medium and low uptake by leaf, respectively. All three substances exhibit both translaminar movement and movement through the xylem while picoxystrobin together with kresoxim-methyl and trifloxystrobin can also be molecularly redistributed by air. Kresoxim-methyl, trifloxystrobin and pyraclostrobin showed only low translaminar ability and no systematic activity trough the vascular system of the plant (Bartlett et al., 2002).

Many research has shown that spore germination in ascomycetes and basidiomycetes and zoospore motility in Oomycetes are the most critical stages for the thrive of the fungal pathogens. The fact that these stages in fungal development are particularly sensitive to QoI fungicides is pivotal for QoI effectiveness (Fernández-Ortuño et al., 2010). Additionally, this heightened sensitivity is attributed to the

fungicide' biochemical mechanism of action such as the disruption of energy production. As these developmental stages are highly energy-demanding, their inhibition effectively prevents successful plant colonization. Therefore, the majority of QoI fungicides exhibit potent activity against zoospore motility in Oomycetes and spore germination in ascomycetes and basidiomycetes when applied during the early stages of infection which is, after infection has occurred but before visible symptoms develop, hence some QoI fungicides are recognized by their eradicant and antisporulant activity. Becker et al. (1981), in their study, suggest that the mode of action of QoI fungicides is single-site. Their fungicidal efficacy stems from their ability to inhibit mitochondrial respiration by binding to the Qo site (outer quinol oxidation site) of the cytochrome bc1 enzyme complex (complex III). The presence of a carbonyl oxygen moiety which is a shared feature of all QoI compounds, plays a crucial role in binding to the enzyme. This respiratory inhibition disrupts electron transfer between cytochrome b and cytochrome c1, halting adenosine triphosphate (ATP) production in the mitochondria of the fungal cell, causing an energy deficiency which ultimately leads to the cell death. The membrane protein complex cytochrome bc1 which is a vital essential for fungal respiration is a main target to QoI fungicide action. In eukaryotes, it comprises 10 to 11 polypeptides with a combined molecular mass of approximately 240 kDa, functioning as a structural and functional dimer. The catalytic core of the enzyme consists of cytochrome b, cytochrome c1, and the Rieske iron-sulphur protein (ISP). Its catalytic mechanism, known as the Q-cycle, requires two distinct quinone-binding sites: the Qo site for quinol oxidation and the Qi site for quinone reduction (Fisher & Meunier, 2008). The precise locations of the quinol/quinone binding sites within the cytochrome b subunit have been identified through X-ray crystallography, using bound inhibitors as models. Detailed insights into the interactions between cytochrome bc1 and various inhibitors, conducted by Esser et al. (2004) have revealed that, QoI fungicides differ by their mode of binding although, they fit within the enzyme pocket on remarkably similar way.

An interesting characteristic of some QoI is their ability to influence the hormonal system of wheat, leading to delayed leaf senescence, enhanced water retention, and increased grain yield (Vincelli, 2012). These fungicides are also associated with beneficial physiological effects on crop yield, attributed to their biological action in promoting net carbon assimilation, interact with plant mitochondrial respiration and enhance nitrate reductase activity in leaf tissues, and improve plant stress tolerance (Glaab & Kaiser, 1999). Such effects have been referred about azoxystrobin, kresoxim methyl and pyraclostrobin which are found to contribute to greater rooting, branching, and bud development leading to increased plant growth and yield (Wu & Tiedemann, 2001; Vincelli, 2012).

ANTIFUNGAL ACTIVITY OF QoI FUNGICIDES

Since their introduction, quinone outside inhibitors (QoIs) have become indispensable in plant disease management programs due to their broad-spectrum efficacy against numerous agriculturally significant fungal diseases. Registered in numerous countries, QoI fungicides are utilized across a wide range of crops, including cereals, turfgrass, grapevines, vegetables, fruits, berries and ornamental plants. Their success can be attributed to the unique properties of individual active ingredients, which typically exhibit one or more of the following attributes: broad-spectrum activity, effectiveness against fungicide-resistant isolates, low application rates, low toxicity to humans, low environmental toxicity, and significant yield and quality benefits (Bartlett et al., 2002). QoI fungicides have driven transformative changes in disease management protocols for various crops. For instance, they provided grapevine growers with the first single active ingredient capable of controlling both powdery mildew (*Erysiphe necator*) and downy mildew (*Plasmopara viticola*). Similarly, in wheat and barley, QoI fungicides especially azoxystrobin have demonstrated superior yield and quality improvements compared to other fungicide classes. Their utility has extended to resistance management programmes for crops like bananas, where they play a critical role in combating persistent and resistant pathogens. Additionally, QoI fungicides are valuable in the maintenance of protected horticultural

crops, particularly in regions like Europe, where the number of available active ingredients has declined due to regulatory compliance. Beyond their foliar applications, QoI fungicides are increasingly used as seed treatments and in-furrow applications to manage soilborne diseases, further expanding their versatility (Bartlett et al., 2002). Among other, mandestrobin is use to reduce the overwintering source of infection in white mould. It is found to possess an inhibitory effect in almost all stages of the pathogen development and especially is appreciated its' activity against sclerotium formation in *Sclerotinia sclerotiorum* in reducing the overwintering source of infection (Hirotomi et al., 2016). Some of the trade names and agricultural applications of QoL fungicides are given in Table 2.

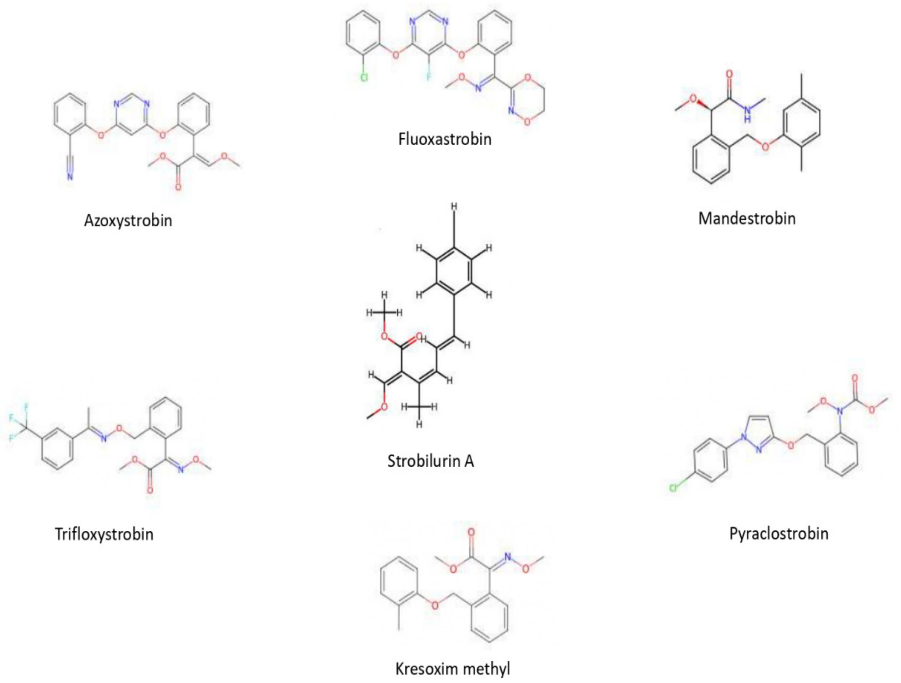

Fig.1 Chemical structural formula of the most important QoI fungicides
Source: Strobilurin A (CSID:4941943, https://www.chemspider.com/Chemical-Structure.4941943.html); Azoxystrobin (CSID:2298772, https://www.chemspider.com/Chemical-Structure.2298772.html); Trifloxystrobin (CSID:32818261, https://www.chemspider.com/Chemical-Structure.32818261.html); Pyraclostrobin (CSID:4928348, https://www.chemspider.com/Chemical-Structure.4928348.html); Kresoxim methyl (CSID:4813314, https://www.chemspider.com/Chemical-Structure.4813314.html); Fluoxastrobin (CSID:9223963, https://www.chemspider.com/Chemical-Structure.9223963.html); Mandestrobin (CSID:34448712, https://www.chemspider.com/Chemical-Structure.34448712.html). Acessed 27.11.2024.

RESISTANCE MECHANISM

Because of their one specific site of action against fungal pathogens, QoIs are highly susceptible to resistance. It is considered that this resistance is mediated by two primary mechanisms such as target site mutations and efflux transporters and a secondary mechanism of QoI resistance which involves alternative respiration such as cyanide-resistant respiration mediated by alternative oxidase. In target side mutations the predominant mechanism of QoI resistance involves mutations in the mitochondrial cytochrome b gene (CYTB), leading to changes in the peptide sequence that inhibit fungicide binding. These mutations are localized to regions of CYTB crucial for ligand binding, particularly amino acid positions 120-155 and 255-280. Key substitutions include G143A (glycine to alanine at position 143), F129L (phenylalanine to leucine at position 129), and G137R (glycine to arginine at position 137) which are all based on single nucleotide polymorphisms in the cytochrome b gene (Gisi et al., 2002; Fisher & Meunier, 2008; Fernández-Ortuño et al., 2008). Research investigations showed that pathogens that carried G143A mutations express high (complete) resistance and are hard to manage.

Anyway, in some cases despite the significant use of the QoIs, the amino acid substitution of the glycine with alanine at position 143 has not been observed even when the QoIs were significantly used in the field. In some species such as *Alternaria solani*, *Pyrenophora teres* and *Puccinia* spp. a non-coding region of DNA (an intron) was observed after the gene that encodes for glycine (FRAC 2011; Grasso et al., 2006). Since this substitution will affect the splicing process and lead to a deficient in cytochrome b, it is considered that the nucleotide substitution with alanine will be lethal and as a consequence resistance based on the G143A mutation is not likely to appear not only in species such as *Alternaria solani*, *Pyrenophora teres* and *Puccinia* spp. but also in other species such as *Uromyces appendiculatus*, *Phakopsora pachyrhizi* and *Hemileia vastatrix* (Fernández-Ortuño et al., 2008). The presence of such an intron was reported in *Monilinia laxa*, *Monilinia fructicola* and *Guignardia bidwellii* (Miessner & Stammler 2010; Miessner et al., 2011). The QoI tolerance of *A. solani* and *P. teres* was indicated by mutations in F129L and/or G137R (Sierotzki et al., 2007) which happen to be of minor importance and according to Semar et al. (2007) have limited impact on the field efficacy of QoIs. Pathogens harbouring F129L or G137R mutations are often overcome by standard field application rates of QoI fungicides. In contrast, the G143A substitution confers high resistance, consistently leading to control failures. This mutation has been documented in over 20 species. Soon after the first QoI fungicides were introduced in 1996, resistant isolates of *Blumeria graminis* f. sp. *tritici* and *Plasmopara viticola* showing G143A mutation were identified (Heaney et al., 2000; Sierotzki et al., 2000b). Other pathogens found resistant to QoI fungicides with this mutation include *Blumeria graminis* f. sp. *secalis*, *Blumeria graminis* f. sp. *hordei*, *Zymoseptoria tritici* (sin. *Septoria tritici*) and its teleomorph *Mycosphaerella graminicola*, *Puccinia recondita*, *Puccinia triticina, Puccinia striiformis*, *Puccinia hordei*, *Rhizoctonia solani*, *Pyrenophora tritici-repentis*, *Pyrenophora graminea*, *Oculimacula* spp., *Rhynchosporium graminicola* (sin. *Rhynchosporium secalis*), *Ramularia collo-cygni*, *Uncinula necator*, *Venturia inaequalis*, Podosphaera leucotricha, *Monilinia* spp., *Stemphylium vesicarium*, *Neofabraea alba*, *N. perennans*, etc. (Tab. 3) (FRAC, 2024b). Despite its clear association with QoI resistance, studying the role of CYTB mutations, particularly G143A, remains challenging due to the mitochondrial origin of the gene and limiting functional genetic studies.

Efflux transporters, specifically members of the ATP-binding cassette (ABC) transporter family and the major facilitator superfamily (MFS), also contribute to QoI resistance by preventing the accumulation of toxic fungicides within fungal cells. These proteins offer broad protection against natural toxins and xenobiotics (Del Sorbo et al., 2000; Stergiopoulos et al., 2003). The first efflux transporter implicated in QoI resistance was MgMfs1, an MFS transporter gene identified in *M. graminicola* (Roohparvar et al., 2007). While overexpression of MgMfs1 is linked to strobilurin resistance, its contribution is typically minor compared to CYTB mutations like G143A.

A secondary mechanism of QoI resistance involves cyanide-resistant respiration mediated by alternative oxidase. This pathway bypasses the cytochrome bc1 complex, sustaining mitochondrial electron transfer and ATP synthesis under fungicide-induced stress (Wood & Hollomon, 2003). However, alternative respiration is energy-inefficient, providing only 40% of the normal ATP yield, which limits its efficacy in planta. Although alternative oxidase may assist fungal survival during late infection stages (e.g., sporulation), it has minimal impact on disease control under the field conditions. Nonetheless, alternative oxidase activity may facilitate the selection of CYTB mutations by reducing reactive oxygen species, potentially promoting the transition from sensitivity to full resistance (Fernández-Ortuño et al., 2008). In addition, pathogens and references linked with the resistance against QoI fungicides according to FRAC are listed in Table 3.

CHALLENGES AND LIMITATIONS

Despite their advantages, QoI fungicides face several challenges that can impact their long-term effectiveness. One of the most significant issues is the development of fungicide resistance in fungal populations. The single-site mode of action of QoIs makes them particularly vulnerable to resistance, as a single genetic mutation in the target site can render

the fungicide ineffective. According to FRAC, resistance has been documented in almost forty fungal species from 26 genera (Tab. 3). To mitigate resistance, best management practices are proposed such as alternating QoIs with fungicides that have different modes of action to reduce selection pressure on fungal populations; to combine QoIs with multi-site fungicides or fungicides from other classes to delay resistance development and to restrict the number of QoI applications per season to minimize the chances of resistance emergence (Fernández-Ortuño et al., 2006).

Another limitation of QoI fungicides is their sensitivity to environmental conditions such as rain fastness which can also vary among products. Heavy rainfall shortly after application may wash off the fungicide and reduce its efficacy (Kovacevik et al., 2001). Additionally, the preventative nature of QoIs requires precise timing, as applications made after infection are often less effective.

Table 2. List of some commercial products based on QoI pesticides and their antifungal activity

Active ingredient	Comercial products	Antifungal activity
Azoxystrobin	PROMESA (Galenika-fitofarmacija, SR); CIROSTROBIN (Sharda Cropchem Limited, India); AMISTAR EXTRA (Syngenta Crop Protection AG, Switzerland); AMISTAR (Syngenta Crop Protection AG, Switzerland); UNIVERSALIS (Syngenta Crop Protection AG, Switzerland); ORTIVA TOP (Syngenta Crop Protection AG, Switzerland); QUADRIS (Syngenta Crop Protection AG, Switzerland); SINSTAR (Sinon EU GmbH, Germany); ABAUND (Syngenta Crop Protection, LLC, USA); BANKIT 25 SC (Syngenta East Africa Limited, Kenya); Heritage (Syngenta Crop Protection, LLC, USA); Protégé (Atticus LLC, North Carolina); AZteroid FC (Atticus LLC, North Carolina); Quadris Top SB (Atticus LLC, North Carolina); Quadris Top SBX (Atticus LLC, North Carolina); Quadris Top (Atticus LLC, North Carolina); Quadris Abound (Atticus LLC, North Carolina); Quilt Xcel (Atticus LLC, North Carolina); Protégé-Allegiance WP (Bayer CropScience, US); Protégé FL Seed applied Fungicide (Bayer CropScience, US); SOYGARD Fungicide Containing Protégé and Allegiance (Bayer CropScience, US), Afiance (), AZOXY TEB, CUSTODIA,	- It is use to control rusts, downey mildews, powdery mildews, rice blast, apple scab, crown rot, damping-off, root rot, white mould etc. in vide variety of crops such as grape vines, cereals, potatoes, apples, bananas, citrus fruit, tomatoes, almons, rice, pistachios, raisins, garlic, turf and ornamental plants. Also, it has a broad spectrum against many important seed and seedling pathogens and it is used in seed treatment, etc.
kresoxim-methyl	Sovran (BASF); Ergon (Various manufacturers); Stroby (BASF); Affinity; Cantos (BASF)	It is used to control apple scab (*Venturia inaequalis*) and powdery mildew (*Podosphaera leucotricha*) in apples; powdery mildew (*Erysiphe necator*) in grapes; leaf spots and molds in citrus fruit; early blight (*Alternaria solani*) and gray mold (*Botrytis cinerea*) in tomatoes and potatoes; Powdery mildew (*Podosphaera xanthii*, *Erysiphe cichoracearum*) in Cucurbits; powdery mildew (*Blumeria graminis*), rusts (*Puccinia spp.*), and leaf spot diseases (*Mycosphaerella spp.*) in cereals; brown patch (*Rhizoctonia solani*), dollar spot, and other turfgrass diseases; white mold (Sclerotinia sclerotiorum) in rapeseed, etc.
mandestrobin	SCLEA flowable (Sumitomo Chemical), INTUITY (Sumitomo Chemical)	ASCOMYCOTA: *Sclerotinia sclerotiorum*, *Monilinia fructicola, Monilinia laxa, Monilinia fructigena, Venturia inaequalis, Venturia nashicola, Diplocarpon mali, Diaporthe citri*; BASIDIOMYCOTA: *Rhizoctonia solani*; OOMYCOTA: *Pythium graminicola*; DEUTEROMYCOTA: *Phomopsis sp., Phomopsis vexanes, Phomopsis fukusii, Alternaria alternata, Botrytis cinerea, Colletotrichum gossypii, Colletotrichum phaseolorum, Colletotrichum simmondsii, Corynespora cassiicola, Cercospora kikuchii, Septoria glycines;* It is used in wide range of crops because of a low risk of phytotoxicity;

pyraclostrobin	SIGNUM 33 WG (BASF SE, Germany), PRIAXOR EC (BASF SE, Germany), REVYCARE (BASF Agro B.V. Arnhem), BELLIS (BASF SE, Germany), BOS (Sharda, Cropchem Limited, India), Pageant (BASF SE, Germany), Intrinsic (BASF SE, Germany), Empress (BASF SE, Germany), Orkestra (BASF SE, Germany), Cabrio (BASF Corporation, USA), Pristine (BASF), PRIAXOR (BASF), PRIAXOR D (BASF)	ASCOMYCOTA: *Botrytis cinerea*, *Alternaria* spp., *Sclerotinia sclerotiorum*, *Mycosphaerella* spp., BASIDIOMYCOTA: *Puccinia* spp., OOMYCOTA: *Phytophthora* spp. *Pythium* spp. DEUTEROMYCOTA: *Colletotrichum* spp. *Rhizoctonia solani*. It is registered for use on a wide variety of crops, including cereals, fruits, berries, vegetables, oilseeds, turf and ornamentals, legumes, etc.
trifloxystrobin	ZATO 50 WG (BAYER CropScience AG, Germany), Flint 500 WG (BAYER CropScience, Australia), Flint Extra (BAYER CropScience US), Stratego (BAYER).	ASCOMYCOTA: *Venturia inaequalis, Venturia pirina, Blumeria graminis, Mycosphaerella* spp., *Sclerotinia sclerotiorum, Podosphaera spp., Erysiphe spp., Monilinia* spp., *Setoria* spp. *Spilocaea spp., Cercospora sojina, Stemphylium vesicarium*; BASIDIOMYCOTA: *Puccinia* spp., *Gymnosporangium fuscum*; DEUTEROMYCOTA: *Alternaria* spp., *Botrytis cinerea, Rhizoctonia solani*; OOMYCOTA: *Plasmopara viticola*; It is registerd in various crops such as brassica vegetables, citrus, cucurbit vegetables, fruiting vegetables, grapes, hops, leafy vegetables, pome fruit, potatoes, rice, root vegetables, stone fruit, strawberries, sugar beets, tree nuts etc.
fluoxastrobin	Teldor (Bayer Cro Science, Australia), EVITO T(Arysta LifeScience, North America)	ASCOMYCOTA: Colletotrichum graminicola, *Colletotrichum truncatum, Setosphaeria turcica, Cochliobolus carbonum, Cochliobolus heterostrophus, Aureobasidium zeae, Septoria glycines, Cercospora spp., Diaporthe phaseolorum, Pyrenophora tritici-repentis, Sclerotium rolfsii, Sclerotinia spp., Monilinia* spp., *Setoria* spp., *Cercospora sorghi*, BASIDIOMYCOTA: *Puccinia* spp., *Phakopsora spp.*, DEUTEROMYCOTA: *Alternaria* spp., *Botrytis cinerea, Rhizoctonia solani*; It is registerd in various crops such as corn, soybean, wheat, pinut, grapes, cherry, plum, apricot, peach, nectarine, strawberry, raspberry, tomatoes, eggplant, lettuce, cornsalad, escarole, garden cress, Bermuda cress, arugula, black mustard, French parsley, Chives, leaf celery, parsley, sage, thyme, basil, rosemary, laurel, estragon, zucchini, pepper, bean, cucumber, oriental plants, etc.

FUTURE PERSPECTIVES

The comprehensive understanding of QoI fungicides underpins their pivotal role in modern agricultural disease management**.** There is no doubt that the future of QoI fungicides lies in addressing their limitations while maximizing their benefits. Since rainfastness is dependent on product formulation, advances in formulation technology can enhance translocation, and extend residual activity overcoming this limitation. For example, encapsulated formulations and nano-carriers are emerging as promising tools to increase the stability and efficacy of fungicides.

Research into the molecular mechanisms of resistance incite the development of the next-generation QoIs with novel modes of action or enhanced binding properties. In addition to this is the fact that there are already several QoI pesticides on the market with such properties like pyribencarb and metyltetraprole registered in Japan with metyltetraprole under review for registration in the European Union. Pyribencarb -is a novel QoI fungicide from the benzyl carbamate group efficient against a wide range of plant pathogenic fungi and active against strobilurin-resistant fungi. Metyltetraprole is another new QoI fungicide possessing a unique tetrazolinone moiety which contributes to its effectiveness against QoI-resistant strains of various pathogen species (Umetsu & Shirai, 2020).

In parallel, integrating QoIs into precision agriculture systems can optimize application timing and dosage, reducing waste and environmental impact.

Biological alternatives, such as biopesticides and microbial antagonists, are increasingly being explored as complementary or substitute options for chemical fungicides (Rocha et al., 2013). While these products currently have limitations in terms of efficacy and consistency, they offer a sustainable approach to disease management and can play a role in reducing reliance on QoIs.

Table 3. List of fungal species with documented resistance to QoI fungicides according to FRAC

Pathogen	Host	Type of resistance	Reference
Alternaria alternata Alternaria tenussima Alternaria arborescens	Pistachio	G143A	Ma et al., 2003; Ma & Michailides, 2004;
Alternaria alternata	Potato, Tomato	G143A	FRAC 2020
Alternaria mali	Apple	G143A	Lu et al., 2003
Alternaria solani	Potato	F129L	Pasche et al., 2002
Ascochyta rabiei	chickpeas	G143A	Delgado et al., 2012
Blumeria graminis f. sp. tritici and hordei	Wheat & Barley	G143A	Sierotzki et al., 2000b
Botrytis cinerea	Strawberries	G143A	FRAC 2020
Cercospora sojina	Soybeans	G143A	Barro et al., 2003
Cercospora beticola	Sugar beet	G143A	Bolton et al., 2012
Cladosporium carpophilum	Almonds	ni	Foerster et al., 2009
Colletotrichum graminicola	Turf grass	G143A	Avila-Adame et al., 2003
Corynespora cassiicola	Cucumber	G143A	Ishii, 2004
Didymella bryoniae	Cucurbit	G143A	Langston, 2002
Erysiphe necator	Grapes	G143A	FRAC 2020
Glomerella cingulata (anamorph *Colletotrichum gloeosporioides*)	Strawberries	G143A	Ishii, 2004
Microdochium nivale Microdochium majus	Wheat	G143A	Walker et al., 2009;
Mycosphaerella fijiensis	Banana	G143A	Sierotzki et al., 2000a
Mycosphaerella graminicola	Wheat	G143A	Fraaije et al., 2005 Sierotzki et al., 2005
Mycosphaerella musicola	Banana	G143A	FRAC 2020
Mycovellosiella nattrassii	Eggplant	G143A	Ishii, 2004
Phaeosphaeria nodorum	Wheat	G143A	Blixt et al., 2009
Plasmopara viticola	Grape	G143A, F129L	Heaney et al., 2000
Pseudoperonospora cubensis	Cucurbits	G143A	Heaney et al., 2000; Ishii et al., 2001;
Pyrenophora teres	Barley	F129L	Sierotzki et al., 2007; Semar et al., 2007;
Pyrenophora tritici-repentis	Wheat	G143A, F129L, G137R	Sierotzki et al., 2007; Stammler et al., 2006;
Pyricularia grisea	Turf grass	G143A, F129L	Vincelli & Dixon, 2002b; Kim et al., 2003;
Pythium aphanidermatum	Turf grass	F129L	Gisi et al., 2002;
Ramularia areola	Cotton	ni	FRAC 2020
Ramularia collo-cygni	Barley	G143A	FRAC 2020
Rhynchosporium secalis	Barley	G143A	FRAC 2020
Rhizoctonia solani AG1.1A	Rice	F129L	FRAC 2020
Sphaerotheca fuliginea	Cucurbits	G143A	Heaney et al., 2000; Ishii et al., 2001;
Stemphylium vesicarium	Asparagus, Pear	G143A	FRAC 2020
Zymoseptoria tritici	Wheat	ni	Hayes et al. 2013
Venturia inaequalis	Apple	G143A	Steinfeld et al., 2002;

ni – no information

CONCLUSION

The QoIs are well known for their great effectiveness against a wide range of plant pathogens, including the most important genera of Ascomycetes, Basidiomycetes, Deuteromycetes, and Oomycetes. They have proven to be an invaluable tool in modern agriculture, offering broad-spectrum activity, systemic properties, and plant health benefits that enhance crop production. However, their reliance on a single-site mode of action makes them susceptible to resistance development, necessitating careful management and integration with other control strategies. Efflux transporter-mediated resistance and alternative respiration play auxiliary roles compared to CYTB mutations in QoIs resistance. The G143A substitution remains the primary driver of resistance in field isolates, emphasizing the

need for integrated resistance management strategies. These may include rotating fungicides with different modes of action, using mixtures to reduce selection pressure, and closely monitoring resistance evolution through robust field studies. While for now, their use has been reduced in certain crops, such as cereals, they continue to play a vital role in many other systems. The commitment to responsible practices and ongoing research will allow the benefits of this valuable class of fungicides to be leveraged for years to come, safeguarding their role in global crop protection. As agriculture faces emerging challenges such as climate change and increasing pathogen resistance, the judicious and innovative use of QoIs which include precise agriculture and the use of advanced technologies in the formulation such as encapsulation and nano formulation, will be essential in the future.

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ФУНГИЦИДИ ИНХИБИТОРИ НА НАДВОРЕШНИОТ КВИНОН, ПЕРСПЕКТИВНА ГРУПА НА ПРОИЗВОДИ ЗА ЗАШТИТА НА РАСТЕНИЈАТА

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Резиме

Стробилурините се класа на фунгициди добиени првенствено од природни супстанци произведени од габи кои се распаѓаат на дрво, особено оние од родот Strobilurus. Откривањето на стробилурините датира од 1970-тите кога истражувачите ги идентификуваа нивните уникатни фунгицидни својства. Оттогаш, различни синтетички аналози на стробилурини се развиени со широк спектар на апликации. Поради нивната активност со широк спектар и ниската токсичност за луѓето и животните, стробилурините станаа едни од најкористените фунгициди ширум светот. Овие фунгициди се ефикасни против различни фитопатогени габи, вклучително и оние кои се одговорни за болести како пепелници, 'рѓи, црна дамкавост, антракнози, септориози, гниење на коренот и сл. Тие најчесто се користат во култури како што се полјоделски култури, овошни култури, јаткасти плодови, зеленчук, бобичести видови, треви и украсни растенија. Нивните системични својства им овозможуваат да се апсорбираат во растителните ткива и да се транспортираат низ растението, обезбедувајќи заштита и на третираните области и на новиот раст што ја зголемува нивната ефикасност и употреба во системите за заштита. Стробилурините се компатибилни со различни производи за заштита на растенијата што ги прави идеални за вклучување во програмите за управување со штетници, плевели и болести. Нивната компатибилност овозможува и мешање во резервоари, намалување на фреквенцијата на апликации и заштеда на време и ресурси за земјоделците. Дополнително, забележано е дека некои стробилурини во одредени услови го поттикнуваат растот на растенијата. Во овој прегледен труд опишани се најзначајните својства на фунгицидите од групата на надворешни инхибитори на квинони. Дополнително во трудот се посветува внимание и на начинот на делување на овие фунгициди, нивните хемиски и фунгицидни својства, резистентноста, недостатоците, можностите и перспективите.

Клучни зборови: *стробилурини, фунгициди, заштита на растенијата, механизам на делување, отпорност.*

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MONITORING OF ACTIVE AND TITRATABLE ACIDITY IN WHITE-BRINED CHEESE DURING RIPENNING PERIOD

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Abstract

This study investigates the dynamics of active and titratable acidity in white-brined cheese throughout its ripening period. Monitoring these parameters is crucial for understanding the biochemical changes that influence the cheese's flavour, texture, and overall quality. Using standard analytical methods, we measured pH and titratable acidity at regular intervals during the ripening process. The results indicated a significant decrease in pH, coupled with an increase in titratable acidity, reflecting the ongoing microbial and enzymatic activities. The findings provide valuable insights for cheese producers to optimize ripening conditions, ensuring consistent product quality. This research highlights the importance of acidity monitoring as a critical control point in cheese production.

Key words: *white-brined cheese, ripening, active acidity, titratable acidity, cheese quality.*

INTRODUCTION

The production of white-brined cheese (WBC), a staple in many Mediterranean and Eastern European diets, relies heavily on the careful monitoring of various biochemical parameters during its ripening period. Among these, active and titratable acidity are critical determinants of the cheese's final quality. Active acidity, measured as pH, provides immediate insight into the hydrogen ion concentration in the cheese matrix, while titratable acidity reflects the total acid content, including both free hydrogen ions and those bound to buffering agents.

Understanding the changes in acidity during ripening is essential for several reasons. Firstly, acidity influences the growth and activity of microorganisms, which are responsible for the biochemical transformations that define the cheese's flavour, texture, and aroma (Fox et al., 2017). Secondly, the balance of acidity impacts the protein and fat stability within the cheese, affecting its consistency and mouthfeel (McSweeney, 2004). Finally, consistent monitoring and control of acidity can help prevent spoilage and ensure safety, thereby extending the shelf life of the cheese (Walstra et al., 2005).

Despite the well-established importance of acidity in cheese ripening, there is a need for more detailed studies focused on white-brined cheese (Makarijoski, 2023). This type of cheese undergoes a unique ripening process in a brine solution, which can significantly influence its acid-base balance (Falih et al., 2024). Therefore, this study aims to systematically monitor and analyse the active and titratable acidity in white-brined cheese throughout its ripening period, providing insights that can help producers optimize their processes and improve product quality.

MATERIAL AND METHODS

To make these examinations, cheese samples of four cheese variants (WBC KS104, WBC KS105, WBC KS110 and WBC KS111) were provided directly from the producers and they were transported under temperature-controlled conditions on 5°C to the Certified Laboratory for Milk and Dairy Product Quality (LB Lact) in Plovdiv, R. Bulgaria. This laboratory is certified

under international standards, ensuring the reliability and accuracy of all testing procedures. Cheese samples for active and titratable acidity as quality parameters were analysed at the 8th, 20th, 30th, 40th and 60th day and were taken from one production series. Methods that were used for this analysis of white-brined cheese were as follow: Active acidity - pH (by using pH–meter, Model MS 2000) and Titratable acidity was determined in °SH, (Sokslet-Henkel, using the method by Caric, 2000). For data processing, we used Microsoft Excel, as a component of Microsoft Office Package. This programme facilitated the tabular representation of data. Additionally, we used the t-test function within Excel to perform statistically significant comparisons between the examined quality parameters (active and titratable acidity) of different cheese variants.

RESULTS AND DISCUSSION

Comparative analysis between experimented white-brined cheese variants for active acidity (pH) dynamics is presented in Table 1.

Table 1. Active acidity (pH) dynamics of examined cheese variants

Active acidity (pH)				
Day	WBC KS104	WBC KS105	WBC KS110	WBC KS111
8	4.90±0.01^{a}	4.81±0.01^{b}	4.74±0.01^{c}	4.88±0.02^{a,d}
20	4.76±0.01^{a}	4.61±0.01^{b}	4.50±0.01^{c}	4.76±0.01^{a,d}
30	4.64±0.01^{a}	4.55±0.01^{b}	4.33±0.01^{c}	4.65±0.01^{a,d}
40	4.54±0.01^{a}	4.45±0.01^{b}	4.16±0.01^{c}	4.58±0.03^{a,d}
60	4.42±0.01^{a}	4.30±0.01^{b}	4.07±0.02^{c}	4.46±0.01^{d}

Differences of values with different superscripts in the same row are statistically significant at level $p<0.05$

Based on the obtained results, it can be concluded that the WBC KS104 variant exhibited the highest active acidity value on the 8th day, measuring 4.90±0.01, compared to the other variants. The lowest active acidity value was observed in the WBC KS110 variant, with a measurement of 4.74±0.02. On the 20th day of the ripening period, the active acidity values for the four variants were ranged between 4.50±0.01 and 4.76±0.02. Further into the ripening period, on the 30th day, the highest active acidity value was recorded for the WBC KS111 variant at 4.65±0.01, while the lowest value was observed in the WBC KS110 variant at 4.33±0.01. On the 40th day, the active acidity values for the four variants ranged from 4.16±0.01 to 4.58±0.03. On the 60th day of the maturation process, all tested WBC variants exhibited a reduction in active acidity compared to the initial state, with values ranging from 4.07±0.03 to 4.46±0.01. The WBC KS111 variant had the highest pH value, which was 0.39 units, 0.16 units, and 0.04 units higher than the WBC KS110, WBC KS105, and WBC KS104 variants, respectively.

Based on the results obtained for the active acidity values of the analysed cheese samples, it can be concluded that during the fermentation process up to the 60th day, there is an expected decrease in pH across the variants, though with varying intensity. This observation highlights the differing activities of lactic acid bacteria from the starter cultures that were used in production process. The addition of starter culture induces normal hydrolytic activity of the enzymes, acting on lactose and proteins. From Table 1, it can also be observed that there is a significant difference in pH values between the four white-brined cheese variants at a significance level of $p<0.05$ for most of the time-periods when the cheese samples were taken for analyses. This can be explained by the different technologies and conditions applied during the cheese production process. The active acidity values obtained align with the findings of Velevski (2015), who reported that the pH of three different white-brined cheese variants produced with different starter cultures was ranged from 4.42 to 4.48 after 60 days ripening period.

Our results are also consistent with those of Balabanova et al. (2017), who found that the average active acidity of Bulgarian white-brined cheese made from cow's milk using different milk-coagulating enzymes was 4.18±0.15%. Similar results were obtained by Ivanov et al.

(2016), who reported an average pH value of 4.2±0.1% in Bulgarian white-brined cheese made from cow's milk after a 45-day ripening period. Slightly higher active acidity values compared to ours were observed by Smiljanić et al. (2014), who reported a pH value of 4.60 units. Chobanova-Vasilevska (2007) reported pH variations in white-brined cheese ranging from 4.50 to 4.80. For feta cheese and other Balkan cheeses, the pH values ranged from 4.2 to 4.8 (Anifantakis & Moatsou, 2006). Higher pH values for white-brined cheese compared to our results were obtained by Lavasani (2014), who studied the effect of different rennet concentrations during cheese production and found active acidity values between 5.0 and 5.04. Similar values were reported by Felfoul et al. (2016), who found an average active acidity of 5.06±0.02 in white-brined cheese made from full-fat milk. Higher pH parameters were also found in Egyptian white-brined cheese, with a value of 4.92±0.10 (El-Aziz et al., 2015).

Comparative analysis between experimented white-brined cheese variants for titratable acidity (°SH) dynamics is presented in Table 2.

Table 2. Titratable acidity(°SH) dynamics in examined cheese variants.

Active acidity (pH)				
Day	WBC KS104	WBC KS105	WBC KS110	WBC KS111
8	69.83±0.02^{a}	74.0±0.40^{b}	74.77±0.06^{c}	73.2±0.40^{d}
20	75.50±0.10^{a}	78.3±0.31^{b}	80.73±0.12^{c}	79.53±0.30^{d}
30	81.37±0.15^{a}	80.26±0.23^{b}	86.13±0.06^{c}	82.7±0.45^{d}
40	82.03±0.11^{a}	82.33±0.21^{a,b}	90.57±0.21^{c}	85.6±0.40^{d}
60	86.52±0.10^{a}	86.53±0.23^{a,b}	101.27±0.12^{c}	88.13±0.23^{d}

Differences of values with different superscripts in the same row are statistically significant at level $p<0.05$

Based on the obtained results, it can be concluded that the WBC KS110 variant exhibited the highest titratable acidity value on the 8th day, measuring 74.77±0.06°SH, compared to the other variants. The lowest titratable acidity value was observed in the WBC KS104 variant, with a measurement of 69.83±0.02°SH. On the 20th day of the maturation process, the titratable acidity values for the four variants ranged between 75.50±0.10°SH (WBC KS104 variant) and 80.73±0.12°SH (WBC KS110 variant). Further during the ripening period, on the 30th day, the highest titratable acidity value was noticed for the WBC KS110 variant at 86.13±0.06°SH, while the lowest value was observed in the WBC KS105 variant at 80.26±0.23°SH. On the 40th day, the titratable acidity values for the four variants ranged from 82.33±0.21°SH to 90.57±0.21°SH. On the 60th day of the maturation process, all tested WBC variants exhibited an increase in titratable acidity compared to the initial state, with values ranging from 86.53±0.23°SH (WBC KS105 variant) to 101.27±0.12°SH (WBC KS110 variant). The highest titratable acidity value was observed in the WBC KS110 variant, which was 14.75, 14.94, and 13.14 units higher than the WBC KS104, WBC KS105, and WBC KS111 variants, respectively. The increased titratable acidity is likely due to the heightened activity of lactic acid bacteria.

Based on the results obtained for the titratable acidity values of the four WBC variants studied, it can be concluded that during the fermentation process up to the 60th day, there is an expected increase in titratable acidity across the variants, albeit with varying intensity. This indicates different bacterial activity from the starter cultures used. The added bacterial cultures induce normal hydrolytic activity of the enzymes, acting on lactose and proteins. From Table 2, can also be observed that there is a significant difference in titratable acidity values between the four white-brined cheese variants at a significance level of $p<0.05$ for most of the tested periods. This can be explained by the different technologies and conditions applied during the cheese production process.

The results obtained in our study are consistent with those reported by Velevski (2015), who found that titratable acidity values in three different white-brined cheese variants produced with different starter cultures ranged from 82 to 86.40°SH after 60 days of fermentation. The values we obtained for the titratable acidity of

the four WBC variants are also within the range reported by Kostova (2013), who found titratable acidity variations from 80 to 96°SH.

Our results are further confirmed by Naydenova et al. (2013), who examined 39 different WBC variants produced in Bulgaria and found that titratable acidity, expressed in Thörner degrees, ranged from 227.6 to 234°T, which converts to 91.01-93.6°SH.

CONCLUDING REMARKS

The monitoring of active and titratable acidity during the ripening period of white-brined cheese provides critical insights into the biochemical processes that determine its quality. This study has demonstrated that significant changes in pH and titratable acidity occur throughout the ripening process, reflecting ongoing microbial and enzymatic activities. These changes are crucial for the development of the cheese's characteristic flavour, texture, and aroma. By understanding and controlling these acidity parameters, cheese producers can optimize ripening conditions, improve product consistency, and ensure high-quality outcomes.

Future research should focus on identifying and characterizing specific microbial strains that influence acidity dynamics and their role in shaping the sensory attributes of white-brined cheese. Investigating the relationship between acidity changes and the development of volatile flavour and aroma compounds would provide deeper insights into the biochemical processes during ripening. Additionally, exploring the impact of varying ripening conditions, such as temperature, humidity, and salinity, on acidity profiles can help refine production protocols. Advanced analytical tools like metabolomics and proteomics could be employed to map the intricate pathways involved in microbial and enzymatic activity. Moreover, developing predictive models that link acidity trends with sensory characteristics and consumer preferences would support the production of consistently high-quality cheese.

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МОНИТОРИНГ НА АКТИВНА И ТИТРАЦИОНА КИСЕЛОСТ КАЈ БЕЛО САЛАМУРЕНО СИРЕЊЕ ЗА ВРЕМЕ НА ЗРЕЕЊЕ

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Резиме

Во ова научно истражување беше следена динамиката на активната и титрационата киселост кај бело саламурено сирење за време на периодот на зреење. Мониторингот на овие параметри е клучен за разбирање на биохемиските промени коишто влијаат на вкусот, текстурата и вкупниот квалитет на овој производ. Користејќи стандардни аналитички методи, беше мерена pH вредноста и титрационата киселост во редовни интервали за време на процесот на зреење. Резултатите покажаа значително намалување на pH вредноста за време на процесот на зреење, а истовремено оваа појава беше пропратена со зголемување на титрационата киселост, што се рефлектира на тековните микробиолошки и ензимски активности. Овие промени беа во корелација со развојот на посакуваните органолептички својства. Наодите обезбедуваат значајни информации за производителите на сирење со цел оптимизирање на условите за зреење, а со тоа и обезбедување доследен квалитет на финалниот производ. Ова истражување ја истакнува важноста на мониторингот на киселоста како критична точка за контрола во производството на сирење.

Клучни зборови: *бело саламурено сирење, активна киселост, титрациона киселост, квалитет.*

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YIELD AND YIELD COMPONENTS IN SOME WHEAT VARIETIES (*Triticum aestivum* L.) GROWN IN KOCANI REGION

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Abstract

The purpose of this research was to study the yield and yield components in seven wheat varieties grown under the soil-climatic conditions of Kochani region. As an experimental material were used six introduced varieties (Rajna, Igra, Epoha, Falado, Grivna and Pobeda) and one domestic variety (Mila). The field trial was conducted in 2022/23 growing season, in randomized block design with three replications. The grain yield, plant height, spike length, number of spikelets per spike, number of grains per spike, grains weight per spike, weight of 1000 grains, number of spikes per m^2 and the biological yield were analysed. Analysis of variance showed high genetic variability and significant differences between the examined varieties for all studied properties. The highest average grain yield was obtained by Falado variety (0,68 kg/m^2). Also, this variety showed the highest average value for the grains weight per spike (2,49 g), weight of 1000 grains (43,0 g) and biological yield (3,1 kg/m^2). The variety Falado was followed by Grivna variety which had the highest value for number of spikes per m^2 (1380 spike per m^2) and number of grains per spike (64,86).

High values of genotypic and phenotypic coefficients of variation (GCV and PCV) were noted for grain yield, biological yield, grains weight per spike, number of grains per spike, spike length, which indicate high variability between tested genotypes. All examined traits showed high heritability. The grain yield was in positive significant correlation with grains weight per spike (r=0,928) and number of grains per spike (r=0,793).

Key words: *wheat, variability, PCV, GCV, heritability, correlation.*

INTRODUCTION

The soft wheat is used for feeding people, domestic animals and for industrial processing. Through wheat processing, products for feeding the larger part of the population are made (Gagro, 1997). Wheat grain is mostly used for high-quality bread and pastry production. With wheat grain processing we get pastry, starch, cookies etc. From all cereal crops, grain wheat has the highest protein quality, suitable composition of mineral matter and vitamins. Besides, the ability to form specific highly-elastic protein complex – gluten and easily digestible starch and proteins, the wheat represents the most important plant food in the world (Peña, 2002).

Today, according to statistics data, 70% of world population consume wheat bread (Vasilevski, 2004). Wheat has a large industrial and economic importance, because the bread made from wheat flour is far better than others by its quality, nutritional and energetic value (Ilievski, 2018).

Wheat has a wide range of distribution, it can be grown in all continents, except for Antarctica. According to FAOSTAT data (2021), the wheat in 2019 was sown on more than 216 million hectares, with average grain yield of 3,5 tons by hectare.

The used of high-yielding varieties and sowing quality seed material are one of the most important factors for reaching high wheat yields (Ilievski, 2015).

The variety as an autonomous genetic, biological and agricultural entity represents one of the crucial factors both in quantity and quality level of production (Dencic et al., 2010). The grain yield growth primarily depends on the variety grown, environmental conditions and the applied technology for growing. The increase of genetic yield potential of new varieties, as well as improvement of other agricultural and

technological properties, enables the increased genetic yield potential in reaching higher production by area unit (Mladenov et al., 2007).

Variety choice for specific agro-ecological conditions is a key factor in realizing high yields. According to Jevtić (1992) the grain yield is influenced by variety about 40%, by applied agro-techniques about 31-40%, and environmental conditions about 20-29%. That means that the variety and the applied agro-technique are nearly equal factors in realizing the wheat yield. High and stable wheat grain yield can be provided by applying the variety technology (Protić et al., 2003).

The genetic potential can be increased in many ways such as: better usage of genetic variability, better usage of solar energy, increasing the number and grains weight per spike, increasing of the total biomass by plant, using the heterosis as a hybrid wheat (Dencic et al., 2010). The selection of suitable location i.e. varieties regionalization will contribute lower variability of grain yields and towards reaching better average results (Madić et al., 2010; Hristov et al., 2012).

Martinčić et al. (1999) have reported that grain yield can be reached by increasing the grains weight with identical number of grains per spike or increasing the number of grains per spike. The grain yield level is determined by three basic components: number of spikes per area, number of grains per spike and the weight of 1000 grains.

The number of grains per spike in an inherent property, but highly influenced by environmental factors. This property often is in negative correlation with the number of spikes per area, which means increasing the density of the crop reduces the number of grains per spike, but not grain weight. The larger number of grains per spike lowers the grain weight and vice versa (Ilieva, 2011).

The weight of grains is also an inherent property little influenced by the environment conditions. According to Martinčić & Kozumplik (1996) the number of grains per spike positive correlated with the variability of grains weight.

The main aim of this research was to determinate the yield and yield components in seven wheat varieties grown in Kocani region.

MATERIAL AND METHODS

Plant material

Seven varieties of winter wheat were used as an experimental material in this study. Five of them were newly created Serbian varieties (Igra, Epoha, Rajna, Falado, and Grivna) under the authority of the, Institute of field and vegetable crops" in Novi Sad, Republic of Serbia. One of the tested varieties was with Macedonia origin (Mila, created under the authorship of the Faculty of Agriculture at "Goce Delchev" University, Stip) and one older Serbian variety Pobeda, used as a standard variety. Pobeda variety was created by the Institute of Field and Vegetable Crops in Novi Sad, Republic of Serbia and it is one of the most dominant varieties in Macedonia, covering significantly larger crop areas compared to other varieties. Pobeda variety had great stability throughout the years regarding its quality and quantity and therefore it is considered as a standard variety in Macedonia, serves as an example in comparative analyses and registration procedures of new wheat varieties.

Field experiment design

The field experiment was set up during 2022/2023 vegetative season, on the plot area with coordinate (41°54'06.0"N 22°21'58.4"E) in village Trkanje, near Kocani, Republic of North Macedonia. The tested varieties were set up in a randomized block design in three repetitions and each of them was placed on an area of 6,25 m^2 (length 5 m and width 1,25 m). On each plot 3750 seeds were sown. The distance between rows was 12 cm and 2 cm inside the row. Certified seed from first generation F_1 was used. The seedling was done my hand in November in 2022.

The soil and agro-technical measurements were applied, i.e. ploughing 35 cm deep as a principal processing and harrowing as a pre seeding processing. Before the field experiment was done, the plot area of 130m^2 was fertilized with 9 kg mineral fertilizer in combination with 15N – 15P – 15K – 10S (9kg/ 0,013ha). For weeds protection, a treatment with „DMA" herbicide

(active ingredient: 2,4 D) was done in the phenophase tillering with a dose of 50 ml/10l of water (in accordance with https://eos.com/crop-management-guide/wheat-growth-stages/).

Before the harvest, measurements of plants' height were made on a random sample of 30 plants for each variety (10 plants of each repetition and variety from the central rows of the plots). The harvest was done in July by hand and from each plot, the samples were taken from 1 m^2 to determinate the grain yield, the total biomass, number of productive spikes in 1m^2, spike length, number of grains per spike, number of spikelets per spike, grains weight per spike and the weight of 1000 grains.

Statistical analysis

All data were statistically processed by descriptive statistics. The analysis of variance (ANOVA) was estimated by SSSP statistical softer (2010). The significant differences between the average values of the tested varieties were scored by LSD (Least Significant Difference) test (Williams, 2010). Heritability (H), phenotypic and genotypic coefficients of variation (PCV and GCV), as well as the linear correlation were calculated by Johnson et al. (1955), Singh & Chaudhary (1977) and Borojević (1986), subsequently.

RESULTS AND DISCUSSION

The data from the tested traits and varieties are given in Table 1. According to the results from the research conducted during and after the harvest of the field experiment, between the examined varieties and the standard variety Pobeda, there were significant morphological-productive differences which is also confirmed by the LSD test and the analysis of the variance. The variety with highest grain yield compared to all tested varieties was Falado (0,68 kg/m^2), followed by variety Grivna (0,66 kg/m^2) and the standard variety Pobeda (0,64 kg/m^2). From all tested varieties Epoha showed the lowest value for grain yield (0,51 kg/m^2). Statistically significant differences were determined between the varieties Epoha, Igra and Falado at level $p<0,05$ and $p<0,01$, whereas the varieties Rajna and Grivna significantly differ only at the level $p<0,05$. From all tested varieties, Rajna variety had the longest stem (91,7cm) while the shortest showed Grivna variety (74,6 cm). From all tested varieties, Macedonian variety Mila had the highest value for spike length (12,08 cm), followed by standard variety Pobeda (10,93 cm) and Falado (10,58 cm). Epoha was the variety with the shortest spike (9,13 cm). For the property spike length, a significant statistical difference at both levels of significances were determined at varieties Epoha, Grivna, Igra and Mila. Variety with the biggest number of spikelets per spike was Mila variety (22,9) whereas the lowest number of spikelets per spike was obtained for Epoha variety (19,73). The variety Grivna had the biggest average value for the number of grains per spike (64,86) which represents significant statistical difference compared to the standard variety at both levels of significance. Also, Falado had high value for number of grains per spike (59,1) and statistically differ compare to all tested varieties. Variety Epoha showed the lowest average value for the number of grains per spike (47,26). Regarding the trait grains weight per spike, statistically significant difference was determined at Epoha variety at both levels of significant. For the same property, the biggest average value was made by Falado variety (2,49 g), whereas the lowest average had the variety Epoha (1,51 g). The highest average value for the property weight of 1000 grains was noticed at Falado variety (43,0 g), followed by standard variety Pobeda (42,66 g). Grivna variety showed the smallest average value for this trait (38,33 g). Regarding the number of spikes per m^2, the highest average had the variety Grivna (1380 spike per m^2), followed by the varieties Epoha (1323,33 spike per m^2) and Igra (1310 spike per m^2). Variety with lowest number of spikes per m^2 was Mila variety (1050 spike per m^2). Similar results have been reported by Areevan at al. (2016), Đurić & Trkulja (2014), Pavkić (2021), Karaman at al. (2022). Only Falado variety had biological yield over 3 kg/m^2 (3,1 kg/m^2), while the lowest average value was received by Igra variety (2,4 kg/m^2).

Grain yield showed high values for the coefficient of phenotype and genotype variability (GCV 44,29 % and PCV 44,41 %) which means that the largest contribution in the total phenotype variability has the genotype i.e. the genetic base of wheat variety. High values

for GCV and PCV were calculated also for the properties: grains weight per spike (GCV 25,59 % and PCV 29,18 %), number of grains per spike (GCV 23,33 % and PCV 24,42 %), spike length (GCV 19,42 % and PCV 20,03 %) and biological yield (GCV 30,75 % and PCV 31,5 %). The high GCV and PCV indicate of wide genetic variability between the examined wheat varieties. High values for GCV and PCV have been reported by Kalimullah et al. (2012), Demelash et al. (2013), Nukasani et al. (2013), Singh & Upadhyay (2013), Choudhary et al. (2020).

Heritability as an inheritance measure of quantitative properties showed high values in most of the examined properties (tab.1). The highest percentage of heritability was noticed for grain yield (99,43 %), but also high values were noticed for the properties: biological yield (95,1 %), spike length (94 %), number of grains per spike (91,27 %), weight of 1000 grains (87,57 %) and number of spikes per m^2 (86,10 %) which indicate high level of inheritance in the examined properties in all wheat varieties. The high percentage of heritability indicate that the properties were less influenced by the environment. The similar results were also found by Petrović at al. (2007), Bhushan at al. (2013) and Kumar at al. (2014).

Table 1. Average values for yield and yield components, genotype and phenotype variability, heritability and least significant differences for tested wheat varieties

Vriety/trait	Grain yield (kg/ m^2)	Plant height (cm)	Spike length (cm)	Number of spikelets per spike	Number of grains per spike	Grains weight per spike (g)	Weight per 1000 grains (g)	Number of spikes per m^2	Biological yield (kg/m^2)
Pobeda	0,64	87,1	10,93	21,56	50,73	2,13	42,66	1160	2,73
Rajna	0,61 *	91,7	10,13*	20,1 *	53,5	2,18	41,66	1176,66	2,83 *
Epoha	0,51 **	89,5	9,13**	19,73 **	47,26	1,51 **	41 *	1323,33**	2,7
Grivna	0,66 *	74,6 **	9,98**	21,83	64,86 **	2,43	38,33 **	1380**	2,61
Igra	0,53 **	79,86 *	10,01**	21,23	49,66	1,94	40 **	1310**	2,4
Falado	0,68 **	85,53	10,58	20,9	59,1 **	2,49 *	43	1226,66*	3,1**
Mila	0,63	84,36	12,08**	22,9 *	56,56 *	2,34	42	1050**	2,46
Average	0,60	84,66	10,40	21,17	54,52	2,14	41,23	1232,37	2,69
$LSD_{0.05}$	0,02	6,72	0,6	1,17	4,58	0,36	1,4	62,79	0,29
$LSD_{0.01}$	0,03	9,4	0,84	1,63	6,41	0,5	1,96	87,85	0,41
GCV (%)	44,29	9,94	19,42	7,12	23,33	25,59	7,72	17,63	30,75
PCV (%)	44,41	12,05	20,03	8,73	24,42	29,18	8,25	19	31,5
H (%)	99,43	68,07	94,0	66,86	91,27	76,92	87,57	86,10	95,1

LSD - Least Significant Difference; GCV - genotype coefficient variability; PCV - phenotype coefficient variability; H – heritability, *statistical significance of differences at p<0,05; **statistical significance of differences at p<0,01

Bhushan at al. (2013) evaluated the grain yield and yield components, as well as genetic variability and heritability using thirty wheat genotypes as an experimental material. According to those authors the highest values of PCV was obtained for the number of grains per plant, followed by harvest index. The lowest values for PCV were reported for the number of spikelets per spike. Highest heritability was revealed for all yield components traits.

In Table 2 are presented the results from analysis of variance (ANOVA). Analysis of variance for all tested properties and wheat varieties confirms that the experiment was set up correctly on a measured surface, and between the examined varieties there was a wide genetic variability, i.e. the varieties were significantly different. Similar findings were also reported by Kumar at al. (2014).

Table 2. Analysis of variance (ANOVA) for yield and yield components in wheat tested varieties

Properties	*SS*	*Df*	*MS*	*F*	*P-value*
Grain yield (kg/m^2)	0.0748	6	0.0124	15.68385	1.73
Plant height (cm)	611.91	6	101.985	18.20895	7.02
Spike length (cm)	15.44	6	2.573	30.13469	1.01
Number of spikelets per spike	20.63	6	3.4383	10.34036	5.44
Number of grains per spike	671.29	6	111.8816	11.57154	3.93
Grains weight per spike (g)	2.05	6	0.3416	17.5645	2.38
Weight per 1000 grains (g)	47.81	6	7.9683	4.290598	0.011631
Number of spikes per m^2	233181	6	38863.49	14.62605	2.6
Biological yield (kg/m^2)	0.989762	6	0.16496	1.034852	0.443643

SS – sum of squares; df - degrees of freedom; MS -mean squares; F -F test; P – critical value

The degree and the intensity of the intercorrelation between tested properties was presented by using linear correlation (Tab. 3).

Grain yield showed high and positive correlation with the weight of grains per spike (r=0,928) and the number of grains per spike (r=0,793), at both level of significance. These results are in accordance with the researchers conducted by Kumar at al. (2014) and Dabi at al. (2016). Significant positive correlation was obtained between spike length and number of grains per spike (r=0,820), but in the same time spike length was negative correlated with number of spikes per m^2 (r=-0,851). The property number of grains per spike was in significant positive correlation with the grains weight per spike (r=0,852), at level of significance p<0,01.

Table 3. Linear correlation coefficient between yield and yield components

Trait	Grain Yield (kg/m^2)	Plant height (cm)	Spike length (cm)	Number of spikelets per spike	Number of grains per spike	Grains weight per spike (g)	Weight per 1000 grains (g)	Number of spikes per m^2	Biological yield (kg/m^2)
Grain yield (kg/m^2)	1								
Plant height (cm)	-0,209	1							
Spike length (cm)	**0,554***	-0,030	1						
Number of spikelets per spike	**0,480***	**-0,566***	**0,820****	1					
Number of grains per spike	**0,793****	**-0,603****	0,273	**0,481***	1				
Grains weight per spike (g)	**0,928****	-0,381	**0,606****	**0,594****	**0,852****	1			
Weight per 1000 grains (g)	0,250	**0,732****	**0,468***	-0,067	-0,310	0,098	1		
Number of spikes per m^2	-0,320	**-0,486***	**-0,851***	-0,426	0,101	-0,303	**-0,739****	1	
Biological yield (kg/m^2)	**0,476***	**0,455***	-0,149	**-0,480***	0,188	0,274	**0,543***	-0,028	1

*statistical significance of differences at p<0,05; **statistical significance of differences at p<0,01

CONCLUDING REMARKS

Based on the results obtained from this research can be concluded that the varieties Falado and Grivna were the most suitable varieties regarding grain yield and yield components compared to standard variety, grown under agro-ecological condition in Kocani. Falado variety had the highest value for grain yield (0,68 kg/m^2), followed by Grivna variety (0,66 kg/m^2), which is by 0,2 kg/m^2 i,e, 0,4 kg/m^2 larger grain yield from the standard variety Pobeda (0,64 kg/m^2). Also, Falado variety showed the highest values for grains weight per spike (2,49 g), weight of 1000 grains (43,0 g) and biological yield (3,1 kg/m^2), while Grivna variety has the largest average

values for number of grains per spike (64,86) as well as number of spikes per m^2 (1380 spike per m^2). Analysis of variance and LSD test showed statistically significant differences between tested varieties and examined properties. Grain yield was in a significantly positive correlation with the number of grains per spike (r=0,793) and grains weight per spike (r=0,928), while highly significant positive correlation was also determined between the other yield components such as: number of spikelets per spike and spike length (r=0,820), the weight of 1000 grains and the plant height (r=0,732), number of grains per spike and grains weight per spike (r=0,852).

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ПРИНОС И КОМПОНЕНТИ НА ПРИНОС КАЈ НЕКОИ СОРТИ НА ПЧЕНИЦА (*Triticum aestivum* L) ОДГЛЕДУВАНИ ВО КОЧАНСКО

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Резиме

Целта на ова истражување беше да се проучат приносот и компонентите на приносот кај седум сорти на пченица во почвено-климатски услови на Кочанскиот Регион. Анализирани се шест интродуирани сорти (Рајна, Игра, Епоха, Фаладо и Гривна) и една домашна сорта (Мила). Истражувањата се реализирани во производната 2022/23 година, во рандомизиран блок-систем со три повторувања. Анализирани се приносот на зрно, висината на стеблото, должината на класот, бројот на клавчиња во класот, бројот на зрна во класот, тежината на зрната во класот, тежината на 1000 зрна, бројот на класови во m^2 и биолошкиот принос. Анализата на варијансата покажа широка генетска варијабилност и значајни разлики меѓу испитуваните сорти за сите испитувани својства. Највисок просечен принос на зрно е добиен од сортата Фаладо (0,68 kg/m^2). Оваа сорта покажа највисоки просечни вредности и за тежина на зрна по клас (2,49 g), маса на 1000 зрна (43,0 g) и биолошки принос (3,1 kg/m^2). Втора најприносна сорта е сортата Гривна, која покажа највисоки вредности и за број на класови на m^2 (1380) и број на зрна по клас (64,86).

Високи вредности на генотипскиот (GCV) и фенотипскиот коефициент на варијабилност (PCV) беа евидентирани за принос на зрно, биолошки принос, тежина на зрна по клас, број на зрна по клас и должина на клас, што укажува на висока варијабилност меѓу генотиповите. Сите испитувани својства покажаа висока херитабилност. Приносот на зрно покажа високо значајна позитивна поврзаност со број на зрна по клас (r=0,793) и тежина на зрна по клас (r=0,928).

Клучни зборови: *пченица, генотипска варијабилност, фенотипска варијабилност, херитабилност, корелација.*

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MICROBIOLOGICAL AND CHEMICAL PROPERTIES OF TRADITIONALLY PRODUCED APPLE, BLACKTHORN, HAWTHORN AND PEAR VINEGAR

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Abstract

The aim of this paper is to determine the quality of vinegars through microbiological and chemical properties using simple chemical and microbiological analysis in different types of vinegar from apple (*Malus sp.*), blackthorn, (*Prunus spinosa* L.), hawthorn (*Crataegus sp.*) and pear (*Pyrus sp.*) obtained in a traditional manner. The amount of acetic acid in vinegar in this investigation varied between 0.72% to 4.5% for the samples.

In apple vinegar, the total number of bacteria amounts to 16 cfu/mL, consists of only acetic bacteria, contains 4.0% acetic acid and has 3.25 pH value. In the samples of blackthorn and hawthorn vinegar, a very small number (6 cfu/mL and 5 cfu/mL respectively) of acetic bacteria was present. Also, a low acidity (pH about 1.0) was found and microscopy revealed Gram (+) rod-shaped asporogenous bacteria (41 cfu/mL in blackthorn and 35 cfu/mL in hawthorn vinegar) and a near absence of acetic bacteria. Hence, samples of blackthorn and hawthorn vinegar were found to undergo no acetic fermentation process at all, meaning the final product is not vinegar. In pear vinegar, the total number of bacteria is 25 cfu/mL and acetic bacteria 16 cfu/mL, it has the highest percentage of acetic acid (4.5%). Yeast cells were observed under the microscope which is characteristic of alcoholic fermentation. Due to it, the process of obtaining the final product goes in an unwanted direction.

A very high negative correlation between pH and acetic acid was determined and between the total number of bacteria and the number of acetic bacteria.

Key words: *Malus sp., Prunus spinosa L., Crataegus sp., Pyrus sp..*

INTRODUCTION

Vinegar, as well as wine, has been known to mankind for many millennia. Ancient winemakers noticed that wine left in an open container becomes sour and turns into a sour liquid with a specific smell. For many years, vinegar was produced without understanding the essence of the process. The first vinegar in history was made from date wine about 7000 years ago in Babylon, Ancient Egypt and Assyria. At that time, vinegar was used as an antiseptic and solvent, it was used as an antidote and general tonic for reducing thirst. There are many myths about the healing properties of vinegar. The vinegar-soaked sponge offered to the crucified Christ was actually a way to ease his suffering. A mixture of apple vinegar and honey was considered an effective remedy for arthritis, although no medical explanation has been found for this.

For centuries, vinegar has been used in food. It was widely used as a spice to improve the taste of hot dishes and to make marinades and spices. Red wine vinegar was used as a salad dressing, and for marinating red meat. White wine vinegar, rice and corn vinegar are combined with chicken and fish dishes and used in baked goods. Malt and cane vinegar are used in various sauces.

In traditional medicine, vinegar is used in its pure form for therapeutic and prophylactic purposes. It is a folk medicine that aids in all kinds of health problems. The most popular is apple vinegar. People used it for therapeutic

purposes, such as weight loss, lowering blood sugar levels, treating respiratory diseases, improving cardiovascular circulation, better digestion, against inflammation of throat, bad breath, various inflammatory processes, high temperature, preventing the feeling of fatigue, etc. Vinegar was an ideal natural supplement for skin care (makes it soft and smooth), helps against warts and dandruff, is a perfect balm for smooth and shiny hair, acts as a natural deodorant, completely neutralizes odours.

Some scientific investigations clearly state the beneficial properties of vinegar such as antifungal, antiviral, antibacterial (due to the low pH value) and antioxidants properties. Vinegar decreases triglycerides, helps with weight loss, controls blood sugar levels, improves cardio-vascular health, prevents hypertension, cardiovascular diseases, cancer, diabetes (type 2), neurodegenerative diseases, osteoporosis, relieves arthritis pain and stomach ache, regulates body pH and detoxifies the body, provides numerous benefits related to skin, digestion and immunity health without any side effects (Sakanaka & Ishihara, 2007; Tripathi, 2023). But, like other acids, vinegar can damage tooth enamel.

Because of its variety and rich history, vinegar attracts the attention of collectors and tasters. High-quality, aged vinegar is considered a good investment, and a collection of original vinegars is valued as much as a collection of rare wines. Vinegar lovers experiment with adding this spice to various dishes, create their own mixes, prepare vinegar at home, and even grow new types of acetic acid bacteria.

As a product, vinegar is obtained from various cereals available in nature, sugar cane, grapes and fruits. Traditional vinegar is produced from regional foods according to well established customs. Different types of vinegar are available on the market. The balsamic vinegar of Modena, Italy is made from the local white Trebbiano grapes. Traditional rice wine vinegar is produced in Asia, coconut and cane vinegar is common in India and the Phillipines and date vinegars are popular in the Middle East, persimmon, sour cherry etc. (Sakanaka & Ishihara, 2007; Singh & Mishra, 2017; Wang et al., 2023).

Vinegar is an acidic liquid with a sharp smell that is obtained through the fermentation process (Najdenovska & Čolo, 2013). In French, the word vinegar actually means "sour wine". Fermentation is done by bacteria. The existence of these bacteria was proved by Louis Pasteur in 1864. In the 70's of the 20th century, there was a revolution in the production of vinegar. It was discovered that acetic acid, which is the main component of vinegar, can be obtained not only by fermentation of wine, must, honey, juices and other liquids containing alcohol, but also chemically from natural gas, industrial waste and products of dry distillation of wood. Vinegar prepared through traditional technology has a special taste and aroma. Vinegars prepared according to special recipes are very popular in American, European and Asian cuisine. In Japan, rice, barley and corn vinegars are made using acidobacteria, which makes them particularly useful. Cherry vinegar is made from a mixture of several wines and stored in wooden barrels for a long time.

In Macedonia, vinegar is traditionally produced from apples and pears. Vinegar from these species of fruit is produced in plastic barrels (wooden barrels were used in the past) by fermenting previously ground fruits (Selamovska et al., 2023). The fermentation process lasts several months (Tešić, 1981; Ziberoski, 2006).

MATERIAL AND METHODS

The examination of the quality of vinegar in this paper included laboratory tests of the microbiological and chemical properties of four different types of vinegar samples: apple (*Malus sp.*), blackthorn (*Prunus spinosa* L.), hawthorn (*Crataegus sp.*) and pear (*Pyrus sp.*) (Figure 1), produced in a traditional manner of production. The samples were taken from different producers who produce vinegar using their own technology (Figure 1 and 2).

Figure 1. Samples of vinegar from fruits: 1-apple, 2-blackthorn, 3-hawthorn, 4-pear (Original photo by O. Najdenovska, 2024).

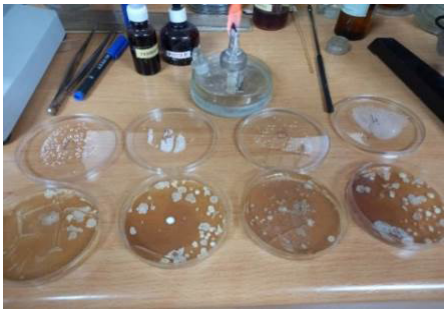

Figure 2. Colonies of bacteria in vinegar in Petri dishes on nutrient medium MPA (Original photo by O. Najdenovska, 2024).

The microbiological analysis of four samples of vinegar was carried out in two iterations. The tested types of microorganisms were cultivated in a thermostat at a temperature of 25 °C. The microbiological analyses included examinations of the total number of bacteria and presence and total number of acetic bacteria (*Acetobacterium*). The analyses were performed in the microbiological laboratory at the Faculty of Agricultural Sciences and Food in Skopje.

The total number of bacteria, as well as, acetic bacteria, were examined on nutrient medium mesopeptone agar (MPA) according to the method of diluting and seeding of selective nutrient medium (Jarak & Djuric, 2004). In the second iteration, the acetic bacteria were examined by the method of diluting and seeding of selective nutrient medium Henneberg (250 mL distilled water, 7.5 g maltose, 2.5 g peptone, 2.5 g yeast extract, 5.0 g agar and 10 mL of ethyl alcohol).

The chemical analyses were performed in two iterations, on 10.12.2023 and 26.02.2024. The measurement of pH in vinegar was performed with a pH meter, while the percentage presence of acetic acid in vinegar was determined volumetrically by 0.1 M NaOH, in the presence of the indicator phenolphthalein. The consumed volume of the base was used to calculate the percentage of acetic acid (OIV-MA-VI-01- Compendium of international methods of analysis for vinegar- Methods of analysis for vinegar). The analyses were conducted in the Oenological laboratory of Institute of Agriculture, Skopje.

Correlation analysis (r.) between determined variables was applied using XLSTAT 2014 software. Data matrix has been introduced using descriptive statistical analysis: minimum, maximum, mean value and standard deviation.

Correlation coefficient (r.) description (LaMorte, 2021) is as follows:

- +1.0 Perfect positive
- +0.8 to 1.0 Very strong positive
- +0.6 to 0.8 Strong positive
- +0.4 to 0.6 Moderate positive
- +0.2 to 0.4 Weak positive
- 0.0 to +0.2 Very weak positive or no association
- 0.0 to -0.2 Very weak negative or no association
- -0.2 to -0.4 Weak negative
- -0.4 to -0.6 Moderate negative
- -0.6 to -0.8 Strong negative
- -0.8 to -1.0 Very strong negative
- -1.0 Perfect negative

RESULTS AND DISCUSSION

According to the results obtained from the microbiological analysis of the total number of microorganisms and acetic bacteria in all four samples of apple, blackthorn, hawthorn and pear vinegar in both iterations, no presence of other types of microorganisms was found except acetic bacteria, yeasts and rod-shaped asporogenous bacteria. In apple vinegar, no other types of bacteria were observed except for acetic bacteria (16 cfu/mL) of the genus *Acetobacter* (Table 1). The determined acidity (pH) of the environment in all samples ranges from 3.12 to 3.96 (Table 2).

Table 1. Total number of bacteria and acetic bacteria (cfu/mL) in apple, thorn, hawthorn and pear vinegar samples.

Samples	Apple vinegar	Blackthorn vinegar	Hawthorn vinegar	Pear vinegar
Total number of bacteria/cfu/mL	16	41	35	25
Total number of acetic bacteria (*Acetobacter)* /cfu/mL	16	6	5	16

Table 2. Chemical analyses for vinegars obtained in December, 2023.

Samples	Acetic acid /%	pH
Apple (*Malus sp*.)	4.00	3.35
Blackthorn (*Prunus spinosa* L.)	0.96	3.68
Hawthorn (*Crataegus sp.*)	0.72	3.96
Pear (*Pyrus sp*.)	4.50	3.12

These tests showed that the samples of blackthorn and hawthorn vinegar contained a low number of acetobacteria (6 cfu/mL in blackthorn and 5 cfu/mL in hawthorn vinegar). Also, in these two vinegar samples, a high quantity of other types of bacteria was determined (41 cfu/mL in blackthorn and 35 cfu/mL in hawthorn vinegar). The obtained pH values were 3.96 in hawthorn, 3.68 in blackthorn vinegar, while 3.35 in apple and 3.12 in pear vinegar. During the first chemical analysis, pear vinegar had the highest percentage (4.50%) of acetic acid (Table 2).

During the determination of the organoleptic properties of the examined vinegar samples, it was determined that the apple vinegar sample had a pleasant, apple smell and taste of vinegar. The smell of the blackthorn vinegar sample did not have a recognizable smell of vinegar, while the smell of hawthorn vinegar had an unpleasant smell. Pear vinegar didn't have a smell recognizable as vinegar but it was not unpleasant, probably due to the presence of yeasts and acetic bacteria. Pear vinegar contains 4.50% acetic acid and had pH value 3.12 (Table 2). The assumption is that the pH value affects the development of yeasts that's why they are present in large quantities (25 cfu/mL) (Table 1).

Acetic bacteria that are obligately aerobic, rod-shaped, Gram negative, asporogenous and motile with polar cilia usually produce 5% to 14% acetic acid (Najdenovska et al., 2013; Najdenovska & Čolo, 2012). Acetic bacteria of the genus *Acetomonas xsylina* produce a small amount of acetic acid, about 4.5%, but they produce a thicker coating - cuticle (Leifson, 1954). In our research, only the apple vinegar sample was observed to form a coating - cuticle.

According to the results of the research of microbiological properties of the vinegar samples in the first iteration (10.12.2023), it was found that the blackthorn and hawthorn vinegar samples contain the lowest quantity of acetic bacteria and a high quantity of total number of bacteria. This condition corresponds to the low percentage presence of acetic acid in vinegar (Table 2). Specifically, the percentage of acetic acid in blackthorn vinegar is 0.96% (0.96 g/100 mL acetic acid), while in hawthorn vinegar it is 0.72% (0.72 g/100 mL acetic acid). Therefore, it was established that the samples of blackthorn and hawthorn vinegar, according to the parameter of amount of acetic acid, the uncharacteristic smell for vinegar, the presence of other Gram-positive asporogenous bacteria and the absence of acetic bacteria (Tešić, 1981), do not represent vinegar.

During the second iteration of the microbiological and chemical analysis (Table 3) of the vinegar samples (26.02.2024), the following properties and parameters were determined:

Table 3. Chemical analyses for vinegars obtained in February, 2024.

Samples	Acetic acid /%	pH
Apple (*Malus sp.*)	4.00	3.25
Blackthorn (*Prunus spinosa* L.)	1.10	3.62
Hawthorn (*Crataegus sp.*)	1.00	3.96
Pear (*Pyrus sp.*)	4.50	3.13

The apple vinegar sample had a completely pleasant taste and smell of apple vinegar, and during microscopy only acetic bacteria were observed in the field of view (Figure 3a,b). In the same apple vinegar, a visible slimy coating from the so-called "mother of vinegar", starter acetic bacteria were observed. Acetic bacteria are able to oxidize sugars and ethanol, producing acetic acid in the fermentation process (Raspor & Goranovic, 2008; Mamlouc & Gullo, 2013; Salieri & Giudici, 2008).

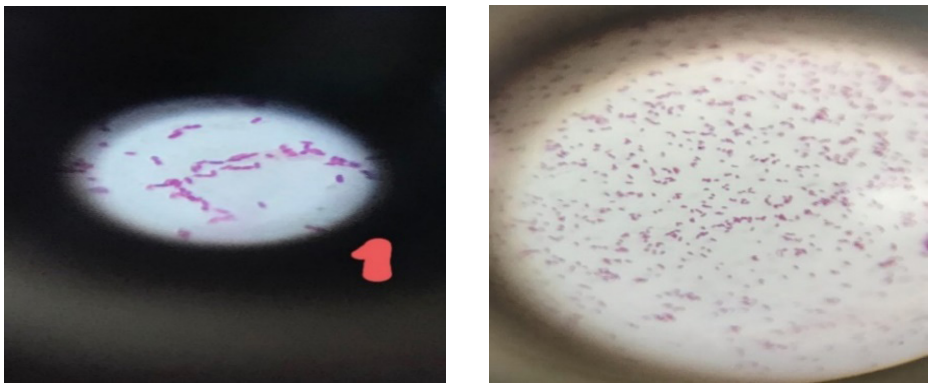

Figure 3. Acetic bacteria in apple vinegar (under microscope).

During the second analysis of the samples, a quantity of 4.0 g/100 mL of acetic acid was determined in the sample of apple vinegar and only the presence of acetic bacteria (16 cfu/mL) (Figure 3) was determined under the microscope. Also, the results obtained for blackthorn and hawthorn vinegar, show that the samples have not a recognizable smell and taste characteristic for vinegar, which was also confirmed through chemical analysis (Table 3), very low content of acetic acid (around 1.0%). Under the microscope, it was found that only Gram (+) rod-shaped asporogenous bacteria (Figure 4,5) dominated, while acetic bacteria were not found, that's why the blackthorn and hawthorn vinegar samples do not represent vinegar according to the established microbiological-chemical parameters.

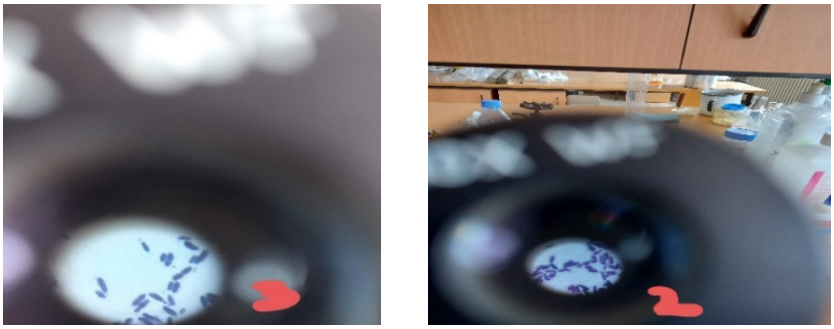

Figure 4. Microorganisms in blackthorn vinegar. Figure 5. Microorganisms in hawthorn vinegar.

During the second microscopic analysis, the sample of pear vinegar, in addition to acetic bacteria, showed yeasts (Figure 6) characteristic of alcoholic fermentation. Another characteristic of the sample of pear vinegar is that it doesn't smell like vinegar, but like pomace, probably due to the presence of yeasts.

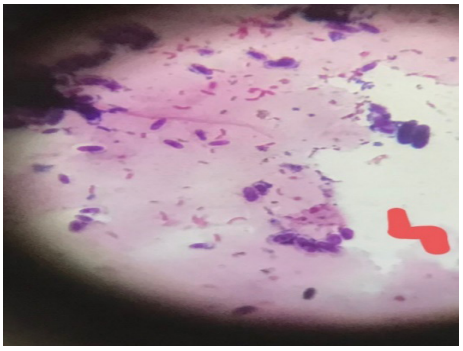

Figure 6. Microorganisms in a sample of pear vinegar

According to legal regulations, vinegar that is commercially available must contain 4-8% acetic acid and low pH value. Vinegar containing 10% acetic acid is corrosive and can cause chemical burns. Spirit vinegar is a stronger form of vinegar that contains about 20% acetic acid. The other chemicals in vinegar depend on its source. Many types of vinegar include added flavourings (sugar, malt or caramel, various spices, etc.) that are added after the fermentation process.

According to the results from the examination, the blackthorn and hawthorn vinegar samples do not comply with the legal regulations (Rules for the quality of vinegar and diluted acetic acid No. 24/1989) for vinegar due to the very low amount of acetic acid (about 1%).

Table 4. Correlation between pH and acetic acid.

Variables	Acetic acid /%	pH
Acetic acid /%	**1**	**-0.9516**
pH	**-0.9516**	**1**

Values in bold are different from 0 with a significance level alpha=0.05.

p-values

Variables	Acetic acid /%	pH
Acetic acid /%	**1**	0.0484
pH	0.0484	**1**

Values in bold are different from 0 with a significance level alpha=0.05

Coefficients of determination (R^2)

Variables	Acetic acid /%	pH
Acetic acid /%	**1**	0.9055
pH	0.9055	**1**

The obtained results presented in Table 4. show a very high negative correlation for pH and acetic acid, when the concentration of acetic acid increases, the pH value decreases and vice versa. In this case, the value of p is 0.0484, which means that there is a statistical significance of the aforementioned correlation dependence between the parameters.

The coefficient of determination R^2 is a statistical value that shows how much the variation of one parameter depends on and it is explained by the variation of another parameter. The higher the coefficient, the higher the dependence. In this case there is a very high coefficient of determination between the studied parameters: acetic acid and pH, when 91% of the variation in the concentration of acetic acid is explained by the pH value and vice versa.

Table 5. Correlation between acetic acid and pH, correlation matrix (Pearson).

Variables	Acetic acid /%	pH
Acetic acid /%	**1**	-0.9370
pH	-0.9370	**1**

Values in bold are different from 0 with a significance level alpha=0.05

p-values

Variables	Acetic acid /%	pH
Acetic acid /%	**0**	0.0630
pH	0.0630	**0**

Values in bold are different from 0 with a significance level alpha=0.05

Coefficients of determination (R^2)

Variables	Acetic acid /%	pH
Acetic acid /%	**1**	0.8779
pH	0.8779	**1**

The results in Table 5 show that there is a very high negative correlation between pH and acetic acid, but somewhat lower compared to the results from the year 2023. When the content of acetic acid increases, the pH value decreases and vice versa. The table shows that there is a very high negative correlation between pH and acetic acid, but somewhat lower compared to the results from the year 2023. When the content of acetic acid increases, the pH value decreases and vice versa. In this case, the value of p (0.063) in year 2024 does not have statistical significance of the aforementioned correlation dependence between the parameters. In this case there is a very high (but somewhat lower in year 2024) coefficient of determination between the studied properties: acetic acid and pH, when 88% of the variation in the concentration of acetic acid is explained by the pH value and vice versa.

Table 6. Correlation between total and acetic bacteria number.

Correlation matrix (Pearson)

Variables	Total number of bacteria/cfu/mL	Total number of acetic bacteria (Acetobacter) /cfu/mL
Total number of bacteria/cfu/mL	**1**	-0.8993
Total number of acetic bacteria (Acetobacter) /cfu/mL	-0.8993	**1**

Values in bold are different from 0 with a significance level alpha=0.05

p-values

Variables	Total number of bacteria/cfu/mL	Total number of acetic bacteria (Acetobacter) /cfu/mL
Total number of bacteria/cfu/mL	**0**	0.1007
Total number of acetic bacteria (Acetobacter) /cfu/mL	0.1007	**0**

Values in bold are different from 0 with a significance level alpha=0.05

Coefficients of determination (R^2)

Variables	Total number of bacteria/cfu/mL	Total number of acetic bacteria (Acetobacter) /cfu/mL
Total number of bacteria/cfu/mL	**1**	0.8088
Total number of acetic bacteria (Acetobacter) /cfu/mL	0.8088	**1**

The results in Table 6 show that there is a very high negative correlation between the total number of bacteria and number of acetic bacteria. When the number of total bacteria increases, the number of acetic bacteria decreases. According to the p value (0.1007), there is no statistical significance of the aforementioned correlation dependence. In this case there is a very high coefficient of determination between the investigated parameters: total number of bacteria and total number of acetic bacteria, when 81% of the variation in total number of bacteria is explained by total number of acetic bacteria and vice versa.

CONCLUDING REMARKS

According to the results obtained from the research, it has been established that the different types of fruits have an influence on the quality and course of vinegar production in a traditional manner.

According to the parameters of the chemical and microbiological analysis, apple vinegar has the best quality, which contains 4.0% acetic acid, pH 3.35 and 16 cfu/mL acetic bacteria.

In the blackthorn and hawthorn vinegar samples, the presence of Gram (+) rod-shaped asporogenous bacteria was determined, that's why the fermentation process does not unfold according to the scientifically established postulates for the production of vinegar. The percentage of acetic acid in blackthorn and hawthorn vinegar is about 1.0%.

Pear vinegar had the presence of acetic bacteria (16 cfu/mL), pH value 3.12, and 4.50% acetic acid. In addition to acetic bacteria, the presence of yeasts has also been determined. Due to the fermentation process unfolded in the direction of alcoholic fermentation, this sample smells of pomace. The process of acetic fermentation should continue to be monitored.

Blackthorn and hawthorn samples do not comply with the legal regulations for vinegar due to the very low amount of acetic acid.

According to statistical analysis, a very high negative correlation between pH and acetic acid was determined. When the content of acetic acid increases, the pH value decreases and vice versa.

A very high negative correlation was found between the total number of bacteria and the number of acetic bacteria. When the number of total bacteria increases, the number of acetic bacteria decreases.

The number of acetic bacteria is negatively correlated with pH and the total number of bacteria.

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МИКРОБИОЛОШКИ И ХЕМИСКИ КАРАКТЕРИСТИКИ НА ТРАДИЦИОНАЛНО ПРОИЗВЕДЕН ОЦЕТ ОД ЈАБОЛКО, ТРНИНКА, ГЛОГ И КРУША

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Резиме

Целта на овој труд е со хемиски и микробиолошки анализи да се утврди квалитетот на примероците од оцет добиен од некои овошни видови: јаболко (*Malus sp.*), трнинка (*Prunus Spinosa* L.), глог (*Crataegus sp.*) и круша (*Pyrus sp.*) добиени на традиционален начин. Количеството на оцетна киселина во мострите варира од 0,72 % до 4,5 %. Во јаболковиот оцет вкупниот број на бактерии изнесува 16 cfu/mL и истиот се состои само од оцетни бактерии, содржи 4,0 % оцетна киселина и има pH вредност 3,25.

Во примероците оцет од трнинка и глог присутен е мал број на оцетни бактерии (6 cfu/mL, односно 5 cfu/mL). Исто така, утврдена е ниска киселост (pH околу 1,0) и микроскопијата утврди грам (+) неспорогени стапчести бактерии (41 cfu/mL кај трнинка и 35 cfu/mL во оцет од глог) и речиси целосно отсуство на оцетни бактерии. Утврдено е дека во примероците оцет од трнинка и глог воопшто не се одвивал процес на оцетна ферментација, што значи дека финалниот производ не е оцет.

Во оцетот од круша присутни се 25 cfu/mL вкупен број на бактерии, 16 cfu/mL оцетни бактерии и во него има најголем процент на оцетна киселина (4,5 %). Во примероците се забележани и клетки од квасци, карактеристични за алкохолна ферментација, поради што процесот за добивање на краен производ оди во несакан правец.

Утврдена е многу висока негативна корелација помеѓу pH и оцетна киселина и помеѓу вкупниот број на бактерии и бројот на оцетни бактерии.

Клучни зборови: *Malus sp., Prunus spinosa L., Crataegus sp., Pyrus sp..*

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WATER QUALITY OF KOSELSKA RIVER AND OHRID LAKE IN THE DALJAN REGION

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Abstract

The aim of this paper is to examine the state and quality of the water from Koselska river, as well as its influence on the quality of water in the coastal zone, more precisely where the mouth of the river flows into the lake. Also, the goal of this research was to look at the nature and degree of possible water pollution, due to anthropogenic influence, based on the results obtained from the testing of microbiological and physio-chemical parameters, which would make it possible to predict and recommend remedial measures and minimize pollution.

To correctly interpret the results of the microbiological analysis of water, physical and chemical tests of water temperature and BOD5 were also performed.

The microbiological examination included testing the composition and dynamics of heterotrophic, psychrophilic and mesophilic bacteria, determining the index of mesophilic and psychrophilic bacteria (M/P), the index of facultative oligotrophic and heterotrophic bacteria (FO/X), as well as the total number of coliform bacteria. It was determined that the representation and abundance of all types of microorganisms is higher in the river water than in the lake water. The total number of bacteria from Daljan region is greater in the summer period compared to other periods of the year. Likewise, consistently high values of the number of coliform bacteria were determined in the two types of water. Based on the results obtained in this research, it was determined that the water from the river affects the quality, health and safety of the water in Ohrid Lake.

Key words: *microbiological properties, bacteria, pollution.*

INTRODUCTION

Ohrid Lake is the largest potable water basin on European soil because of its clean water. Also, Ohrid Lake is a unique ecosystem in which relict plant and animal species live. The lake is important in relation to tourism, fishing, and the economy. Ohrid Lake has an oligotrophic character but feels the burden of anthropogenic influence. It is threatened by intense mineral and organic pollution that would cause a change in the water quality.

The need for continuous monitoring and research, to assess the water quality, is imperative in the protection of the lake and the maintaining of the water quality at the level of safe drinking water. Moreover, the research on the water quality of the Koselska River, as one of the biggest tributaries of the lake, is one of the important indicators of the condition of the water and the direction for further action in the protection of the lake.

Microorganisms are constantly present in aquatic ecosystems and actively participate in the transformation of organic matter and biochemical processes in the water, and thus in the functioning of the ecosystem as a whole. Microorganisms are the first indicators of eutrophication (Najdenovska & Čolo, 2012; Matzinger et al., 2007).

The microbiological research and categorization of the water in this paper indicates possible organic and faecal pollution. The assessment of the water quality, as well as the

physiochemical parameters, gives a complete picture of the condition of the defined aquatic ecosystems and will enable the assessment of the anthropogenic influence on the Koselska River and Ohrid Lake in Daljan region, near the mouth of the river into the lake.

Similar tests related to water ecology in our country were carried out on parts of Black River, near Bitola, for determination of *Escherichia coli* and *Enterococcus* (Blazhevska et al., 2024). Also, the investigation conducted in the Strumica valley, located in the south-eastern part of North Macedonia, where the content of arsenic, iron, manganese and some other polutants were investigated in Strumica River by group of authors (Kovacevic et al., 2021).

The purpose of this research is to evaluate the condition and quality of the water in the Koselska River through microbiological and physiochemical indicators and its influence on the quality of the water in Ohrid Lake in Daljan region, near the river mouth.

MATERIAL AND METHODS

The scope of this paper was determination of water quality of the Koselska River and Ohrid Lake from Daljan region, at the mouth of the river into the lake, where on the riverbed of Koselska River before the lake and the coastal zone of Ohrid Lake in the immediate vicinity of the river's mouth. The examination of water quality in the paper included testing the number and dynamics of bioindicator microorganisms from a sanitary and ecological aspect, as well as testing the physiochemical parameters of the water, such as water temperature and BOD5. (Strickland & Parsons, 1972). Microbiological analyses of the water included testing of heterotrophic (saprophytes and organotrophs) and oligotrophic bacteria and their ratio (FO/X), mesophilic and psychrophilic bacteria and their ratio (M/P), and total coliform bacteria (Govedarica & Jarak, 2003).

Physico-chemical analysis

For the physico-chemical parameters, the classification was carried out according to the Decree on the categorization of waters (Official Gazette of Macedonia No. 18/99) and the legal regulations on the categorization of surface waters. An assessment of the quantitative-trophic state is calculated according to the trophic state index (Carlson, 1977) while the classification is performed according to the trophic scale (Aizaki et al., 1981).

Sampling for physico-chemical and microbiological research from the coastal zone was carried out based on standard limnological methods, using a Routier bottle (APHA-AWWA-WPCF 1980, 2005). The samples were taken in spring, summer, autumn and winter periods.

Two physico-chemical parameters in Koselska River were examined: water temperature (measured with a reversible depth thermometer Welch, 1948), and biochemical oxygen demand (BOD5), determined according to the Winkler's method (Bether 1953, APHA-AWWA-WPCF 1980).

Microbiological analyses

Microbiological analyses were carried out at the Department of Microbiology at Hydrobiological Institute - Ohrid. Liquid and solid media were used to demonstrate the number of microorganisms, general and selective media according to standard methods: APHA-AWWA-WPCF (2005).

The M/P index has been used as an indicator of bacterial contamination from a hygienic point of view. Facultatively oligotrophic bacteria are identified on 10% MPA medium (mesopeptone agar diluted 10 times) and incubated for 5-7 days at 22 °C.

The FO/X index (facultative oligotrophs/ heterotrophs) has been used as an indicator of the state and quality of water from a broader ecological perspective.

Heterotrophic (saprophytes, organophytes) bacteria are determined on a standard substrate of mesopeptone agar (MPA) by incubation for 48 hours at 35-37 °C for mesophilic bacteria (M) and 5-7 days at 20-22 °C for psychrophilic bacteria (P).

Total coliform bacteria are determined by the petri plate method (with the dilution method) on a selective chromogenic medium.

RESULTS AND DISCUSSION

The results obtained from the research of the water quality from the Koselska River and the mouth of the river in Ohrid Lake from Daljan region are presented in this paper. For this paper, microbiological analyses of the examined water from the Koselska River and Ohrid Lake were conducted. Microbiological parameters refer to the microbiological characteristics of the examined water.

Heterotrophic bacteria are important for the biological decomposition of organic substances that play a key role in the release and recycling of nutrients as well as in the processes of natural water self-purification (Najdenovska & Čolo, 2012).

The high value of the total number of heterotrophic bacteria indicates water rich in organic substances, susceptible to bacterial decomposition (Najdenovska & Čolo. 2012). In these researches, according to the obtained results (Figure 1), it was established that there is a constant presence of heterotrophic mesophilic bacteria in the water from the two investigated localities (Koselska River and Ohrid Lake, near Daljan region) and that the number of these bacteria is higher in the water from the river than the lake near Daljan, in all four annual periods. The number of bacteria in the Koselska River is highest in the autumn period (1592 bact./mL) and in the summer period (1249 bact./mL). In Daljan region, the number is relatively low, with the lowest value being determined in the spring period (52 bact./mL), and the highest in the summer season (1104 bact./mL). This situation indicates the strong influence of the river on the lake coastline, especially in the summer period (Figure 1). A similar situation was noted in the research by Lokoska (2015) during the summer season in the localities of the coastline zone of Ohrid Lake. The increased number of mesophilic bacteria in the water in the summer and autumn period is due to the increased water temperatures in the aquatic ecosystems and the increased content of soluble organic substances, as pointed out by Sipkoska Gashtarova et al. (2008) in the research conducted in the Streževo reservoir.

Psychrophilic heterotrophic bacteria, which grow at temperatures up to 220 °C are considered natural aquatic bacteria and their numbers are a good indicator of nutrients available for bacterial nutrition (Malecka & Donderski, 2006).

Figure 2 presents the results for the number of heterotrophic psychrophilic bacteria in the water samples from Koselska River and the water from the coastal zone near the river mouth in the Daljan region.

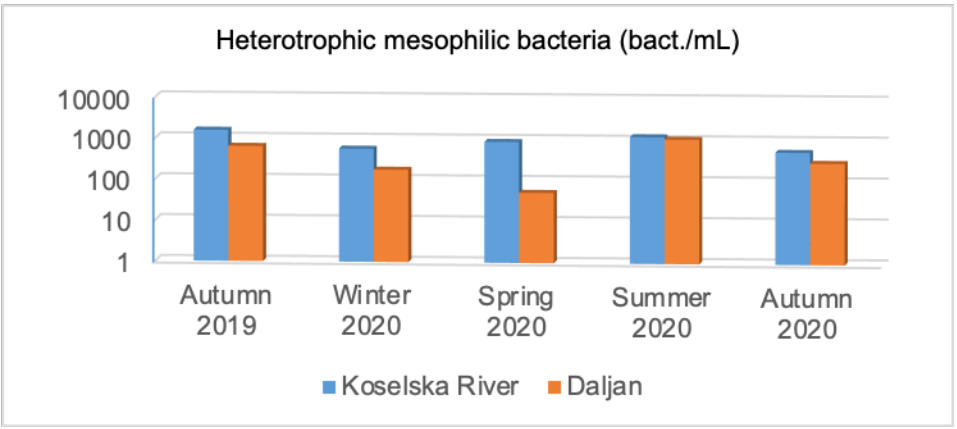

Figure 1. Heterotrophic mesophilic bacteria.

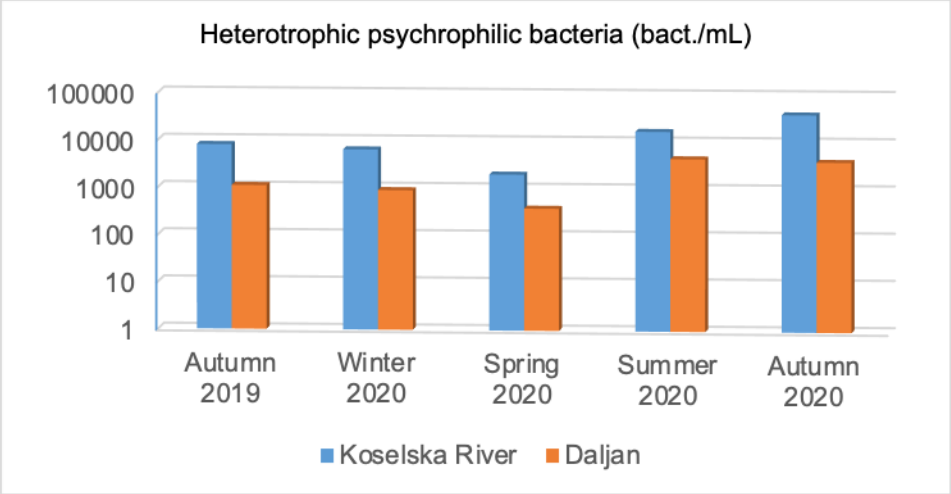

Figure 2. Heterotrophic psychrophilic bacteria.

It can be noted that the psychrophilic heterotrophic bacteria in the Koselska River have higher values in the summer and autumn periods (Figure 2). The highest number was observed in autumn (39840 bact./mL). The same dynamics of the number of bacteria in relation to the season were also determined in the water from the Daljan coastline, with maximum values in the autumn period (3980 bact./mL), and minimum values in the spring (384 bact/mL). In general, it is observed that the values of the number of psychrophilic bacteria in the water from Koselska River are significantly higher than in the water from the coast near the river mouth in Daljan region.

Significantly higher values of psychrophilic bacteria in the research by Lokoska, (2015) were recorded in the water samples from the tributaries of Ohrid Lake than in the lake coastline, specifically in the summer period. The greater representation of psychrophilic bacteria in water in the summer period is due to the decrease in precipitation and the increase in the concentration of organic substances in the water ecosystem (Stojanova, 2012).

The values of the M/P index as an indicator of bacterial pollution from a hygienic point of view, from Koselska River in all examined periods of the year, are presented in Table 1. M/P values below 0.3 are considered favorable, while elevated values indicate a pathogenic effect, that is, of a dominantly higher representation of mesophilic heterotrophic bacteria that are potentially pathogenic for humans and animals (Petrović et al., 1998).

Table 1. M/P index in water from the Koselska River.

Koselska River	Autumn 2022	Winter 2023	Spring 2023	Summer 2023	Autumn 2023
Mesophilic bacteria (bact./mL)	1592	496	1056	1249	560
Psychrophilic bacteria (bact./mL)	6240	5040	2432	16900	39840
M/P index	0.255	0.091	0.434	0.074	0.014

The M/P index for the Koselska River has the highest value for the spring of 2023 (0.434), a period of intense rains and a greater inflow of water into the river bed from melting snow and drainage water. Similar research was done by Blazevska (2016) in the water of the Crna River, into which the Fifth Canal flows. At the junction of the canal and the Crna River, the M/P index was 5.154, which indicates poor water quality at the point where the Fifth Canal flows into the river. The author explains the reason for this situation with the transfer of waste materials from the canal directly into the river. Table 2 shows the value of the M/P index for the water from the coastline near Daljan.

Table 2. Index of M/P in water from Daljan coastline.

Littoral Daljan	Autumn 2022	Winter 2023	Spring 2023	Summer 2023	Autumn 2023
Mesophilic bacteria (bact./mL)	624	227	52	1104	320
Psychrophilic bacteria (bact./mL)	1024	794	384	3920	3980
M/P index	0.609	0.286	0.135	0.282	0.080

The values of M/P in the water from the Daljan coastline are lower than 0.3 in all investigated seasons, which indicates clean coastal waters, where psychrophilic heterotrophic bacteria dominate over mesophilic ones. An exception to this situation was observed in autumn 2022 (Table 2), when the index has the highest value, i.e. 0.609, but also the number of mesophilic heterotrophic bacteria in this period is significantly higher compared to the other seasons in the samples taken from Daljan.

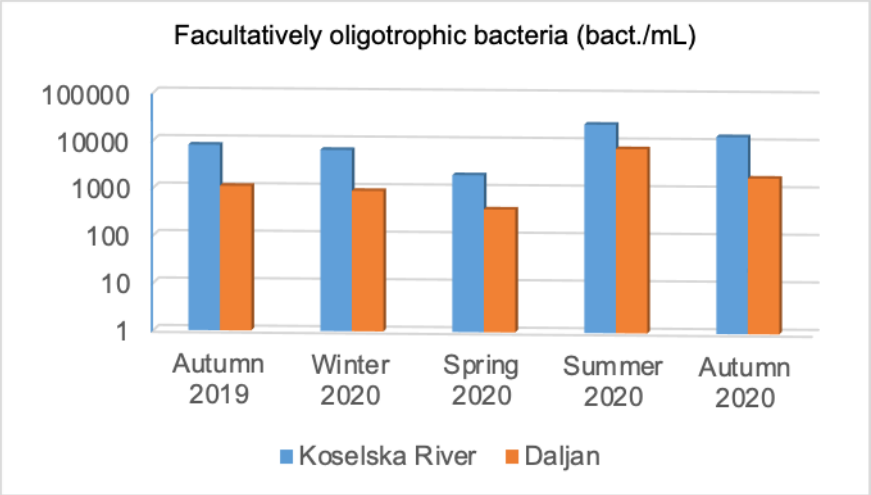

Figure 3. Facultatively oligotrophic bacteria.

In this paper, the representation of facultative oligotrophic bacteria that can live in environments rich in organic matter, but also in an environment with a low concentration of organic matter, was examined. The dominance of these bacteria in water is characteristic of unpolluted water, water with a low concentration of nutrients, that is, water with an oligotrophic character, and water in which self-purification processes are expressed.

Figure 3 shows the values for the number of facultative oligotrophic bacteria in the Koselska River and the Ohrid Lake from Daljan region. The graph presented on Figure 3 shows a higher presence of the examined bacteria in the water from the Koselska River than in the water from the Daljan region. The highest values in the river were determined in the summer of 2023 (24320 bact./mL) and the fall of 2023 (13920 bact./mL) and the lowest value in the spring of 2023 (1692 bact./mL).

The water quality from Daljan region follows the values of the number of facultative oligotrophic bacteria in the river. Hence, for this locality, higher values were measured during the summer of 2022 (7488 bact./mL) and the fall of 2022 (1890 bact./mL). The lowest value of these bacteria was determined in the spring of 2022 (304 bact./mL) both in the water from the Koselska River and from Daljan region.

Veljanoska Sarafiloska et al. (2019), found in their research that in the coastline zone of Ohrid Lake and Prespa Lake, a higher number of facultative oligotrophic bacteria was recorded during the summer period, which is in direct correlation with the increase in nutrients (total nitrogen and phosphorus) and the organic matter in the water. These groups of bacteria are more prevalent in the water from the rivers in the catchment area of Ohrid Lake (Velgoška, Čerava,

Koselska and Sateska) than in the lake coastline near their mouths (Lokoska, 2019).

The relationship between facultative oligotrophic bacteria and heterotrophic bacteria (FO/X index) is an indicator of the state and quality of water from a wider ecological perspective. The data obtained from this parameter indicate a greater or lesser ability of self-purification of water ecosystems in the Koselska River (Table 3) and Daljan region (Table 4).

Table 5 presents a proposed characterization for self-purification of water depending on the numerical values obtained for FO/X, based on which the results of the research in this paper will be interpreted.

Table 3. FO/X index for water from the Koselska River.

Koselska River	Autumn 2022	Winter 2023	Spring 2023	Summer 2023	Autumn 2023
Facultative oligotrophic bacteria (bact./mL)	6752	3648	1692	24320	13920
Heterotrophic bacteria (bact./mL)	6240	5040	2432	16900	39840
Index FO/X	1.08	0.72	0.70	1.44	0.35

Table 4. FO/X index for water from Daljan region.

Daljan	Autumn 2022	Winter 2023	Spring 2023	Summer 2023	Autumn 2023
Facultative oligotrophic bacteria (bact./mL)	1038	897	304	7488	1890
Heterotrophic bacteria (bact./mL)	1024	794	384	3920	3980
Index FO/X	1.02	1.13	0.79	1.91	0.48

According to the obtained numerical values for the FO/X - index (Table 5), in this paper, it was established that the values of the index are higher than 1 in Koselska River in autumn 2022 and summer 2023, which according to the proposed classification indicate a satisfactory ability of self-purification of the ecosystem. In the other seasons, the value of the index - FO/X is lower than 1, which means that in those seasons, the studied aquatic ecosystems have a weak self-purification ability.

Table 5. Proposed characterization of the self-purification ability of water depending on the FO/X index (according to Petrovic et al., 1998).

FO/X index value	Self-purification ability of water
<1	Weak
>1	Satisfactory
>1	Good

Coliform bacteria as indicators of faecal water pollution were also investigated in this paper. The value of coliform bacteria in the water from Koselska River and Ohrid Lake was recorded as constantly high. The total number of coliform bacteria in the water from the Koselska River ranges from 18000 bacteria/mL in autumn 2022 to 24100 bacteria/mL in all other seasons.

In lake water from the coastline near Daljan region, the total number of coliform bacteria is significantly lower compared to the river water. The maximum value was recorded in the summer of 2023 (9000 bacteria/mL), and the minimum in the autumn of 2022 (2000 bacteria/mL).

According to Lokoska (2015), faecal pollution is especially pronounced in the lake littoral and near the river mouths, in the summer period. This is due to the introduction of municipal and industrial wastewater rich in pollutants, organic substances, and bacteria into

the rivers.

According to the results of the research in this paper on the values of the temperature of the water in Koselska River, it was determined that it is constantly lower than the lake water. The temperature difference between the maximum and minimum values in the Koselska River is lower (9.3 °C), compared to the lake water (12.3 °C).

Also, it was stated that in the summer season, the BOD5 was recorded, for both localities in the research. The highest value for Koselska River is 3.19 mg/L, while for the Daljan region, it is 3.20 mg/L. This condition corresponds to the intensive processes of mineralization of the organic matter, for which the high temperatures in the summer period represent a stimulating factor.

CONCLUDING REMARKS

Based on the research results obtained in this paper, it has been established that The increased number of heterotrophic bacteria (mesophilic and psychrophilic) during the summer period is in direct correlation with the warming of aquatic ecosystems and the increase of pollutants of organic origin under the influence of people, especially in the summer months, in the peak of the tourist season, when the load is even more pronounced. The quality of water from Koselska River is generally II class, except in summer and autumn 2023 when it is III class. The water from the coastline at Daljan during the research period indicates I class water, and in spring 2023 the water belongs to I class.

According to the value of the M/P index for the Koselska River and the littoral near Daljan, in all the studied seasons it is low, i.e. all below 0.3, which indicates clean waters that are not dominated by mesophilic heterotrophic bacteria. The values for the FO/X index in the water in the lake littoral, during the investigated seasons, have a higher value than the river and according to the proposed classification, generally indicate a satisfactory ability of self-purification of the lake water. In the water of the Koselska River, the number of coliform bacteria is significantly higher compared to the lake water from Daljan region, which indicates an obvious strong anthropogenic influence on the river water, as well as on the water from the coastal zone of the lake. Temperature is the main driver of the intensity and dynamics of the basic processes of circulation of matter in nature and as such affects the biological activity of living organisms.

The water temperature in Koselska River and the lake near Daljan during the research period in this paper is directly proportional to the climatic characteristics of the area, although the water temperature of the Koselska River is lower (9.3 °C) than the lake water (12.3 °C). That is why two periods are differentiated: a period of heating of aquatic ecosystems (spring-summer) and a period of cooling (autumn-winter). The highest biochemical oxygen consumption for five days in the two studied localities was determined during the summer season (3.19 mg/L), and the lowest values for both localities were recorded during the winter period (0.906 mg/L).

In general, it was found that during the researched period, the number of the tested bacteria was higher in the water from Koselska River than in the coastal zone near the Daljan region and in summer period compared to other seasons, which is due to the increase in the temperature of the water in the river and lake ecosystem and directly affects the processes of mineralization of organic substances in water systems.

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КВАЛИТЕТ НА ВОДАТА ОД КОСЕЛСКА РЕКА И ОХРИДСКО ЕЗЕРО ВО РЕГИОНОТ НА ДАЉАН

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Резиме

Целта на овој труд е да се испита состојбата и квалитетот на водата во Коселска Река, како и нејзиното влијание врз квалитетот на водата во крајбрежниот појас, поточно каде устието на реката се влева во езерото. Исто така, целта на ова истражување беше да се погледне природата и степенот на можното загадување на водата поради антропогено влијание, врз основа на резултатите добиени од тестирањето на микробиолошките и физичко-хемиските параметри, со што би се овозможило да се предвидат и препорачаат корективни мерки и минимизирање на загадувањето.

За правилно толкување на резултатите од микробиолошката анализа на водата, извршени се и физичко-хемиски испитувања на температурата на водата и БПК5. Микробиолошкото испитување опфати тестирање на составот и динамиката на хетеротрофните, психрофилните и мезофилните бактерии, одредување на индексот на мезофилни и психрофилни бактерии (М/П), индексот на факултативни

олиготрофни и хетеротрофни бактерии (FO/X), како и вкупниот број на колиформни бактерии. Утврдено е дека застапеноста и изобилството на сите видови микроорганизми е поголемо во речната вода отколку во езерската вода. Вкупниот број на бактерии е поголем во летниот период во однос на другите периоди од годината. Исто така, во двата вида вода беа утврдени постојано високи вредности на бројот на колиформни бактерии.

Врз основа на резултатите добиени во ова истражување, беше утврдено дека водата од реката влијае на квалитетот, здравјето и безбедноста на водата во Охридското Езеро.

Клучни зборови: *микробиолошки карактеристики, бактерии, загадување.*

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Pectobacterium carotovorum subsp. *carotovorum* - CAUSAL AGENT OF SOFT ROT OF PEPPERS PRODUCED IN THE STRUMICA REGION

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Abstract

Pectobacterium carotovorum subsp. *carotovorum* (formerly *Erwinia carotovora* subsp. *carotovora*) is a plant pathogen that causes soft and stem rot diseases in several economically important vegetables such as carrot, cabbage, cucumber, eggplant, garlic, onion, pepper, potato, radish, sweet potato, squash and tomato, where the disease can be detected in the field, transmit, storage and market.

Agricultural producers face many challenges in trying to grow crops and ensure stable, high-quality yields. The risk factors involved in agricultural production include climatic conditions, the occurrence of diseases, pests, and weeds. During our field observation, we examined the production of pepper, its economic and nutritional value, and the factors contributing to its decline.

Our primary focus is the present status of diseases in pepper, specifically the occurrence of *Pectobacterium carotovorum* subsp. *carotovorum*, the causative agent of bacterial soft rot. *P. carotovorum* subsp. *carotovorum* is a well-known plant pathogen that causes severe soft rot disease in various crops, resulting in significant economic loss.

The aim of this study is to highlight the economic importance of the bacterial agent responsible for soft rot in peppers grown in the open fields in Strumica region. For this purpose, standard bacteriological tests were performed. These tests confirmed the pathogenic, morphological, biochemical, physiological, and growth properties of the pathogen, compared with the control strain KFB85 from the Republic of Serbia.

Key words: *diseases, pathogen, bacteriosis, plant bacteria, agricultural producing.*

INTRODUCTION

North Macedonia is a country located in the central part of the Balkan Peninsula, with ideal topographic and climatic conditions for agricultural production. Since ancient times, food production has been the primary means of livelihood in this country. Food production continues to be the subject of numerous and ongoing research worldwide.

Pepper (*Capsicum annuum* L.) is an annual, herbaceous plant of *Solanaceae* family. It originates from Brazil and it is one of the most widely cultivated crops in North Macedonia. Due to its nutritional value and distinct taste, this crop has become an indispensable part of the daily diet. Pepper is a vegetable rich in nutrients, carbohydrates, minerals, and vitamin C, with a concentration as high as 260 mg/100g. It also contains B vitamins and secondary metabolites, with capsaicin being the most abundant (Bosland & Votava, 2012).

In our country, these vegetables are produced both in open fields and greenhouses. The main agricultural regions in North Macedonia are Strumica, Gevgelija, Valandovo, Skopje, Bitola, and Kumanovo. The regions of Strumica, Gevgelija and Valandovo are particularly known for peppers, tomatoes and cucumbers production (Krsteska et al., 2022).

In 2019, the total arable area in North Macedonia was 519,452 ha, with 8,460 ha in the Strumica region, 8,730 ha in the Stip region, and 17,873 ha in the Skopje production region. Annually, 185,452 t of pepper are produced, with nearly 50% coming from the southeastern

region of the country. The most widely cultivated pepper types are kapija type, long (banana) type and different types of hot varieties (Statistical Office of the Republic of Macedonia, 2020).

The high demand and intensive production of pepper bring a series of risk factors, such as the occurrence of diseases, pests and weeds. The occurrence of plant diseases, specifically bacteriosis, is the subject of research in this paper.

Pectobacterium carotovorum subsp. *carotovorum* is the causal agent of bacterial soft rot in pepper and many other plants (Opara & Asuquo, 2016). Interest in studying this bacterium arose after the massive damage it caused to potatoes in Germany. The discovery of *Pectobacterium carotovorum* subsp. *carotovorum* occurred between 1878 and 1900 and it is regularly present in crops cultivated in North Macedonia (Mitrev, 2001; Pejcinovski & Mitrev, 2007).

Pectobacterium carotovorum subsp. *carotovorum* is a phytopathogenic, rod-shaped bacterium with dimensions of 0.5–0.8 × 1.3 mm. It is anaerobic and gram-negative. The arrangement of its flagella is peritrichous, indicating its high mobility. It does not form spores. When isolated on a nutrient medium, the bacterium forms shiny, round, smooth, gray-white colonies, noticeable after 24 hours of incubation (Jee et al., 2020; Lee et al., 2013).

Pectobacterium carotovorum subsp. *carotovorum* hydrolyzes gelatin and esculin, produces hydrogen sulfide, and has the ability to reduce nitrates. The bacterium is catalase-positive and oxidase-negative and produces phenylalanine deaminase, urease, arginine dehydrolase, lecithinase, and phosphatase. One of the most characteristic features of this pathogen is its ability to secrete pectolytic enzymes, which disorganize, soften, and lead to the complete decomposition of plant tissues (Charkowski, 2018; Gašić et al. 2014).

The bacteria typically enter plant tissue through wounds caused by using agrotechnical measures, rain, hail and insects. The most severe infections caused by *Pectobacterium carotovorum* subsp. *carotovorum* typically occur following hailstorms due to the extensive injuries they inflict (Arsenijević, 1988; Arsenijević & Obradović 1996; Ivanović et al., 2009).

MATERIAL AND METHODS

In July and August 2019, we collected symptomatic and asymptomatic pepper samples, from the open fields and greenhouses in Strumica region. The isolates were taken from pepper plants surveyed both in open fields and greenhouses (Figure 1).

Isolation of the pathogen was performed the same day as sample collection. The collected peppers were washed under tap water and then dried at room temperature. Next, small pieces from the symptomatic tissue (the margins between healthy and diseased tissue) were cut and homogenized with sterile deionized water (SDW). After waiting approximately for 5 minutes, the suspension was streaked onto Petri dishes containing nutrient agar (NA) and nutrient agar enriched with sucrose (NAS). The plates were incubated at 26°C for 48 hours.

Small, round, milky-white bacterial colonies that grew on the nutrient agar medium were selected as representatives. Fresh bacterial colonies were then used for further identification of the pathogen (Figure 2). The control strain KFB 85 from the Republic of Serbia was used for comparison. After bacterial colonies characteristic of the desired pathogen developed, some of them were selected for further analysis.

Five isolates, coded as Pcc 5, Pcc 7, Pcc 8, Pcc 13 and Pcc 20, were good types for future research from all collected samples.

Figure 1. Symptomatic pepper fruits collected from the Strumica region.

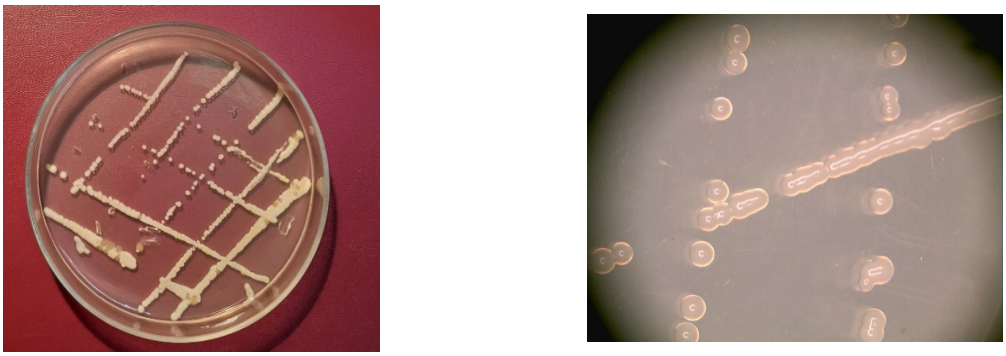

Figure 2. Pure bacterial colonies on NAS medium.

The main virulence determinants of soft-rotting *Pectobacterium* species are cell wall-degrading enzymes, primarily pectinases and cellulases. Identification of the strains was performed using bacteriological tests such as Gram's reaction, hypersensitivity on tobacco, tolerance to 5% and 7% NaCl, oxidase, catalase, pectolytic tests on potato and carrot, and hydrolysis of aesculin (EPPO Bulletin, 2023).

The ability to macerate the host tissue distinguishes this bacterium from many others. The pectolytic activity of the isolated *Pectobacterium* strains was assessed using potato and carrot fruit tissues (Figure 3). Potato tuber and carrot slices were washed with sterile deionized water (SDW), and holes with diameter of 60-70 mm were made. The holes were filled with fresh bacterial suspension (10^9 CFU/ml) and placed in sterile Petri dishes. Sterile distilled water was used as a negative control, while the KFB85 strain as positive control.

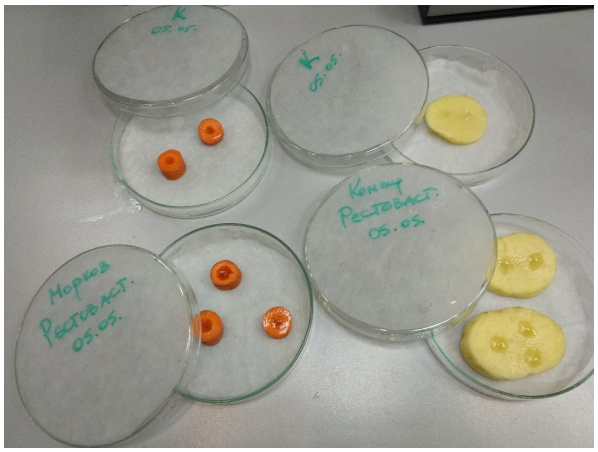

Figure 3. Assessment of pectolytic activity on potato tuber tissue and carrot root tissue.

Pectobacterium carotovorum subsp. *carotovorum* induces a hypersensitive response in tobacco plants. To confirm this, we performed a tobacco sensitivity test by injecting a bacterial suspension (10^9 CFU/ml) into the interveinal tissue of a tobacco leaf. The inoculation procedure is shown on Figure 3. The inoculated tobacco plant was incubated at 25-26°C, and the hypersensitive response was recorded after 24-48 hours (Figure 4).

Figure 4. Inoculated tobacco plant ready for incubation at 25-26°C in UNILAB.

RESULTS AND DISCUSSION

During July and August 2019, symptomatic pepper fruits were collected to confirm the presence of *Pectobacterium carotovorum* subsp. *carotovorum*. The symptomatic fruits exhibited watery tissue, mostly near the plant stem, and some produced an unpleasant odour. Bacteriological and pathogenic tests for our isolates were conducted and they were compared to the positive control strain KFB85. These tests confirmed the pathogen as *Pectobacterium carotovorum* subsp. *carotovorum*. This gram-negative bacterium is oxidase-negative, catalase-positive, and capable of hydrolysing aesculin. It demonstrates pectolytic activity on potato tuber and carrot root tissue and induces a hypersensitive reaction in tobacco leaves (Figure 5 and 6). Additionally, the bacterium demonstrates tolerance to 5% and 7% NaCl (Table 1).

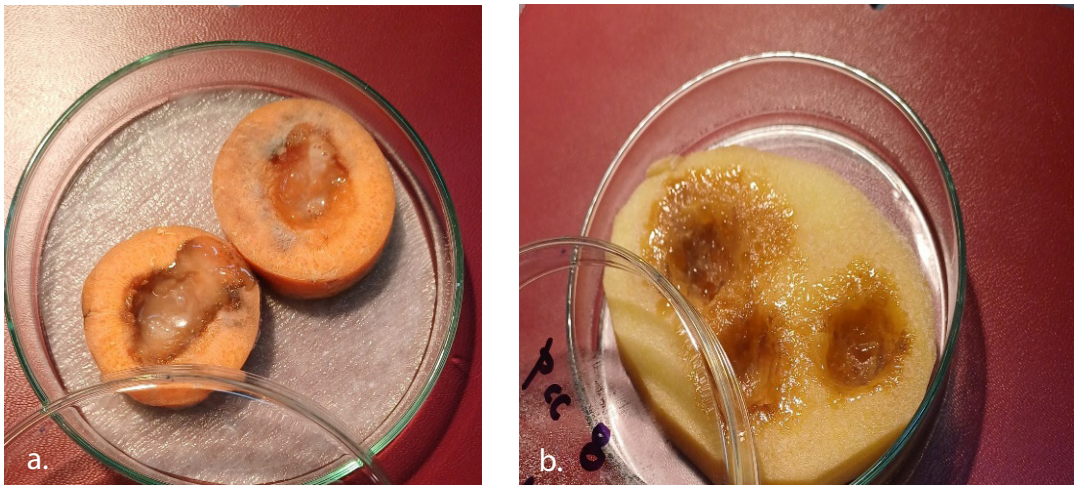

Figure 5. Results of bacterium pectolytic activity on a. potato tuber tissue b. carrot root tissue.

Figure 6. Hypersensitive response (HR) on tobacco leaves 48h after inoculation *Pectobacterium carotovorum* subsp. *carotovorum*.

Table 1. Biochemical and physiological characteristics of *Pectobacterium carotovorum* subsp. *carotovorum* strains and KFB85 as a control strain.

Properties	New isolated strains in UNILAB*					Control strain KFB 85 **
	Pcc 5	**Pcc 7**	**Pcc 9**	**Pcc 13**	**Pcc 20**	
Gram reaction	-	-	-	-	-	-
Activity of oxidase	-	-	-	-	-	-
Activity of catalase	+	+	+	+	+	+
Tolerance of 5% NaCl	+	+	+	+	+	+
Tolerance of 7% NaCl	+	+	+	+	+	+
Hypersensitive reaction (HR) on tobacco	+	+	+	+	+	+
Pectolytic activity on potato	+	+	+	+	+	+
Pectolytic activity on carrot	+	+	+	+	+	+
Hydrolysis of aesculin	+	+	+	+	+	+

+= positive reaction
- = negative reaction
*new isolated strains in UNILAB: Pcc5, Pcc7, Pcc9, Pcc13, Pcc20
**control strain KFB 85 (obtained by personal communication with A. Obradović, Republic of Serbia)

CONCLUDING REMARKS

The global demand for food requires intensive production, and science continues to advance in pursuit of this goal. The aim of this research is to emphasize the importance of constant monitoring to reduce the occurrence of phytopathogenic diseases. The detection and accurate identification of harmful plant pathogens are essential for improving plant disease control strategies (Kulukovska et al., 2021). Additionally, this study seeks to highlight the presence of *Pectobacterium carotovorum* subsp. *carotovorum* in our country.

It is important to note that *Pectobacterium carotovorum* subsp. *carotovorum* poses a serious threat to pepper production, particularly in the Strumica region, the largest vegetable production region in the Republic of North Macedonia, because the rapid spread of this bacterium leads to severe economic losses in the fields.

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Pectobacterium carotovorum SUBSP. *carotovorum* - ПРИЧИНИТЕЛ НА БАКТЕРИСКО ВЛАЖНО ГНИЕЊЕ КАЈ ПИПЕРКАТА ОДГЛЕДУВАНА ВО СТРУМИЧКИОТ РЕГИОН

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Резиме

Pectobacterium carotovorum subsp. *carotovorum* (порано познат како *Erwinia carotovora* subsp. *carotovora*) е растителен патоген кој предизвикува болести на влажно гниење на стеблото кај неколку економски значајни градинарски култури како: морков, зелка, краставица, модар патлиџан, лук, кромид, пиперка, компир, ротквица, сладок компир, тиква и домат, каде болеста може да се открие на терен, да се пренесе и да се складира.

Земјоделските производители се соочуваат со многу предизвици во обидот да ги одгледуваат земјоделските култури и да обезбедат стабилни, висококвалитетни приноси. Ризичните фактори вклучени во земјоделското производство ги вклучуваат климатските услови, појава на болести, штетници и плевели. За време на нашето теренско набљудување, предмет на истражување беше производството на пиперка, неговата економска и хранлива вредност и факторите кои придонесуваат за нејзиниот пад.

Нашиот примарен фокус е сегашниот статус на болести кај пиперката, особено појавата на *Pectobacterium carotovorum* subsp. *carotovorum*, предизвикувач на бактериско влажно гниење. *P. carotovorum* subsp. *carotovorum* е добро познат растителен патоген кој предизвикува сериозна болест на меко гниење кај различни култури, што резултира со значителна економска загуба.

Целта на оваа студија е да се истакне економската важност на патогенот одговорен за влажното гниење на плодовите пиперки одгледувани на отворено во Струмичкиот Регион. За таа цел беа направени стандардни бактериолошки тестови. Овие тестови ги потврдија патогените, морфолошките, биохемиските, физиолошките и својствата на раст на патогенот, во споредба со контролниот вид KFB85 од Република Србија.

Клучни зборови: *болести, патоген, бактериоза, растителни бактерии, земјоделско производство.*

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Professional paper

ERADICATING VARROA MITES (*Varroa destructor*) BY SPRAYING CLOVE TEA

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Abstract

This study is a field experiment of using clove tea as an effective treatment against varroa mites (*Varroa destructor*), by spraying the tea directly on all the frames in the beehive. Two experiments were conducted, one for 14 weeks, with an application of clove tea once every 2 weeks and the other for 8 weeks with an application once per week. In both experiments after 6 applications, the tea-treated varroa-infected beehives were completely varroa-free, concluding complete eradication.

Keywords: *clove tea, Varroa mites, Varroa destructor, honey bee, organic bee treatment.*

INTRODUCTION

Varroa mites (*Varroa destructor*) are parasites that affect the honey bee (*Apis mellifera*), which causes great damage to bee colonies. Synthetic miticides have been used to control varroa infestations. However, some problems arise from the use of such synthetic chemicals. The usage of synthetic miticides creates mites resistant to the chemicals (Rodriguez-Dehaibes et al., 2005), and it contaminates honey, propolis, and wax (Ruffinengo et al. 2002). In addition, bee queen exposure to synthetic miticides during development negatively affects the queen's reproductive health (Rangel &Tarpy 2015). Another study has also found that synthetic acaricide exposure affects bee grooming, the natural removal of mites by the bees scraping them off, it delayed and reduced the overall duration of grooming behavior (De Matos et al., 2017).

An alternative treatment of varroa by using organic compounds is becoming attractive for beekeepers. Essential oils have proven to be effective and safe in treating bee colonies for varroa mites (Skinner et al., 2001), even if the essential oils are administered orally by adding them to the sugar feed are still safe for the bees in the right doses (Ebert et al. 2007).

A study shows that the treatment of bee hives by the method of cotton soaked in clove essential oil had a significant effect on the average mortality of varroa mites (Kadhim et al. 2021). One study showed that clove oil causes a 60% mortality rate in *V. destructor* mites at a dosage of 1.0 µl for 48 hours (Li et al., 2017). Another study indicates that clove oil has an effect of 96% mite mortality on varroa mites with topical applications of eight dilutions of 0.75 mg/vial (Gashout & Guzman-Novoa 2009). Also, a residue of clove oil after treatment was discovered in beeswax during a two-week period under semi-field conditions, which indicates a long-term effect of the oil (Girisgin et al., 2014). Essential oil fumigation and evaporation treatment can effectively control mites in adult bees, but it cannot penetrate the cell capping, therefore it does not control mites in brood cells.

All these above-mentioned studies focus on using essential oils as fumigants or for evaporation. Essential oils do not mix in water; therefore, no consistent and measurable direct spraying method could be applied because the concentration of oil ejected by the sprayer will be random. Moreover, essential oils are usually more expensive than commercial synthetic

miticides. Thus, using clove essential oil is not easily applicable in a consistent matter with a spraying method. However, clove flower buds are relatively inexpensive and easily available, and making clove tea for spraying application is a simple and approachable method for the common beekeeper.

MATERIAL AND METHODS

40 grams of clove buds (*Syzigium Aromatcum*) were placed in 4 liters of hot water and kept covered with a lid until the solution cooled down. The solution (tea) was filtered and poured into a spray bottle. Whole cloves were used to make the solutions because crushed or powdered cloves tended to clog the spray head, caused by residue in the solution.

Field Experiment 1.

One first initial field experiment was done in the Municipality of Makedonski Brod in Macedonia on 12 honey bee colonies in a Langstroth 10 frames hives (one box/floor) of *Apis melisa Macedonica*: 6 varroa infested, 6 non-infested. All six groups were placed in six different locations in the Municipality, at least 15 km apart. Brood was present in the hives with 4 to 5 frames.

Group one - varroa infested, treated with cloves tea.
Group two - varroa infested, treated with water (placebo)
Group three - non-infested, treated with cloves tea.
Group four - non-infested, treated with water (placebo).
Group five - non-infested, control group not treated with anything.
Group six - varroa infested, control group not treated with anything.

The application was done by individually splashing each frame of the hive with the solution (mist) on both sides, applying 4 applications (splashes) per side. The treatment was done in the late afternoon, to allow for more bees to be home in the hive while applying the treatment. The application and inspection were done once every two weeks with a total period of 14 weeks, 8 applications in total. The experiment started on the 1st of March 2023 and ended on the 7th of June 2023.

Anti-varroa floor- Screen bottom board was used in the hives. Anti-varroa floor is a modified bottom board with a screen to allow mites to fall to a metal board or on the grass below the hive. The fallen dead mites on the metal board were counted to calculate mortality. Dead bees' mortality is impossible to accurately count in the field because bees clean the hive and take out the dead bees outside the hive.

Field Experiment 2.

A second larger scale field experiment was conducted in the Municipality of Makedonski Brod in Macedonia starting from the 21th of June, until 16th of August for a total of 8 weeks.

18 honey bee colonies in a Langstroth 10 frames hives (one box/floor) of *Apis melisa Macedonica*: The two groups were placed in two different locations in the Municipality, at 40 km apart. Brood was present in the hives with 4 to 5 frames.

Group one – 9 hives of varroa infested, treated with clove tea.
Group two – 9 hives of varroa infested control group, not treated with anything.

The methodology and other parameters were the same as the first initial experiment, except that the clove tea was applied once per week over a period of 8 weeks. Any sprayed solution (even distilled water) may cause some varroa mites to dislodge from the bees (Elzen et al. 2001). After spraying the water (placebo) it dislodged a small number of the mites from the bees, but it did not kill them. However, in the case of the clove tea, after applying it to the bees, direct contact made mites dislodge from the bees, significantly more than the water placebo, and directly kill and immobilize the mites within 10-25 minutes. Clove tea-treated hives did not experience any evident decrease in bee population or diminished overall bee health.

RESULTS AND DISCUSSION

Field experiment 1

The mite-infested (Group 1) after treatment, Hive 1.1. increased its bee population, it had 6 frames nested with bees at the beginning of the experiment and in week 10 it had 10 frames nested with bees, and hive 1.2. from 7 frames full of bees to 9 frames full of bees. In addition, a calming effect on the bees was observed after the application of the clove tea, and a significant reduction in speed of movement and reduced irritability was noted. The complete results list of dead mites from the treatment with clove tea are shown in table 1.

Table 1. Number of varroa mites dead after treatment, first experiment.

Group	Week 0	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14
H. 1.1	506	190	50	10	2	1	0	0
H. 1.2	600	252	66	37	5	0	0	0
H. 2.1	5	4	10	20	19	32	27	30
H. 2.2	3	6	12	15	22	37	40	47
H. 3.1	0	0	0	0	0	2	0	0
H. 3.2	0	0	0	0	0	0	0	0
H. 4.1	0	0	0	0	0	2	1	5
H. 4.2	0	0	0	0	0	0	0	0
H. 5.1	0	0	0	0	0	0	0	0
H. 5.2	0	0	0	0	0	0	0	0
H. 6.1	11	9	15	25	42	30	39	48
H. 6.2	8	11	7	21	36	47	35	51

Group one - varroa infested, treated with cloves tea. Hive 1.1. After the first application, an estimated 50% of the mites in the hive were eradicated. After 10 weeks and 6 treatments, all signs of Varroa mites vanished in the hive. Hive 1.2. In the first application an estimated 60% of the mites in the hive were eradicated, after 8 weeks all signs of varroa disappeared in the hive.

Group two - varroa infested, treated with water (placebo). In Hive.2.1. and Hive2.2. The number of Varroa mites found dead gradually increased, but that is because the overall live mites population increased in the hive, and most of the deaths are from other causes, not the water treatment.

Group three - non-infested, treated with clove tea. Hive 3.1. No mites were present at the beginning of the experiment. However, in week 10 and week 12, a few dead mites were accounted for. This is assumed that some bees got the infestation from outside the hive. In weeks 12 and 14 there were no dead or live mites in the hive, complete eradication of the newly appeared infestation was achieved. Hive 3.2. No varroa presence was noted during the study period.

Group four - non-infested, treated with water (placebo). Hive 4.1. At week 10, this colony developed varroa infestation, with an increase in counted dead mites as a result of increased overall mite presence in the hive. Hive 4.2. No varroa presence was noted during the study period.

Group five - non-infested, control hives not treated with anything. No mites dead or alive were observed during the experiment.

Group six - varroa infested, control hives not treated with anything. The hives had a gradual increase of counted dead mites due to an expanded overall varroa infestation.

Field experiment 2

Clove tea had the same effect on varroa mites as in the first experiment. Achieving complete eradication of all varroa mites in the treated hives after 6 applications, with no varroa presence by week 7 (Group H 1. 1-9). Two of the treated hives achieved complete varroa eradication by week 6 (5 applications). At the beginning of the Experiment all the hives had 7-9 frames nested with bees. After 4 weeks all clove tea treated hives had 10 frames nested with bees.

In the control group, the hives had a

gradual increase of counted dead mites due to an expanded overall varroa infestation (Group H 2. 1-9). Bee population reduced in the control group to 6-7 frames nested with bees by week 8. The complete results list of dead mites from the treatment with clove tea are shown in table 2.

Table 2. Number of varroa mites dead after treatment, second experiment.

Group	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
H 1.1	581	129	49	19	5	1	0	0
H 1.2	650	150	40	11	2	0	0	0
H 1.3	470	190	60	20	7	2	0	0
H 1.4	510	202	51	17	3	1	0	0
H 1.5	490	120	20	11	9	5	0	0
H 1.6	610	131	59	13	8	2	0	0
H 1.7	548	180	66	29	11	4	0	0
H 1.8	628	93	39	10	3	0	0	0
H 1.9	575	102	50	21	9	2	0	0
H 2.1	6	12	10	19	24	30	29	32
H 2.2	8	10	14	11	16	15	21	24
H 2.3	5	4	11	10	9	23	20	19
H 2.4	3	9	18	20	19	24	31	35
H 2.5	9	15	12	31	29	31	27	41
H 2.6	12	21	15	19	25	30	31	37
H 2.7	5	11	14	21	19	23	20	26
H 2.8	7	12	21	25	31	36	43	46
H 2.9	9	17	29	18	35	34	41	40

Discussions

This experiment of spraying clove tea shows a potential organic approach to completely eradicating varroa mites' presence in bee hives. Clove residues in the honey are unavoidable after so many treatments. However, according to the World Health Organization clove residues in food pose no danger to consumers. Therefore, honey with clove residue is safe for consumption. It can be argued that it is even beneficial for the consumer, because of clove's health benefits to the human body (Parle & Khanna, 2010). The method is labour intensive, requiring individual application on all frames in the hive, as opposed to placing one soaked cloth in essential oil for evaporation within the hive. However, the results were much more effective with this clove tea spraying method because it completely eradicated the *Varroa* mites after 6 applications in the treated hives. In addition, clove buds' tea is far less expensive than clove oil, it is also easily obtained and available in common groceries.

CONCLUDING REMARKS

This study indicates that spraying clove tea on bee hive frames is effective in eradicating varroa in infested bee hives. However, the study has several weaknesses that need further investigations. Additional studies are required for the long-term effects on honey contamination, larvae health, queen reproductive capacity, etc. This experiment focused only on the mortality rate of varroa mites by spraying clove tea. This experiment shows that clove tea eradicates/kill varroa mites. Future studies can prove at what cost does it that, and if there are any side effects or other health issues to the bee colonies by its application.

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ИСКОРЕНУВАЊЕ НА ВАРОА КРЛЕЖ (VARROA DESTRUCTOR) СО ПРСКАЊЕ НА ЧАЈ ОД КАРАНФИЛЧЕ

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Апстракт

Оваа студија е теренски експеримент со користење на чај од каранфилче како ефикасен третман против вароа крлеж (*Varroa destructor*), со директно прскање со чајот на сите рамки во кошниците со пчели. Беа спроведени два експеримента, еден за 14 недели, со нанесување чај од каранфилче еднаш на секои 2 недели, а другиот за 8 недели со нанесување еднаш неделно. Во двата експеримента веќе по шестата апликација од третманите, заразените пчелни семејства третирани со чај беа целосно без варола, заклучувајќи целосно искоренување.

Клучни зборови: *чај од каранфилче, вароа крлеж, вароа деструктор, медоносна пчела, органски третман на пчели.*

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INFLUENCE OF VARIETY AND VINTAGE ON THE BASIC PHUSICO-CHEMICAL COMPOSITION OF SMEDEREVKA AND VRANEC WINES

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Abstract

In this study, Smederevka and Vranec wines from *V. vinifera* L. *cv*., the most important and dominant varieties in Macedonia, have been produced in 6 (six) consecutive years applying traditional fermentation methods, in order to study the influence of variety and vintage on the general basic wine quality. The physico-chemical parameters that confirm the basic wine quality have been determined, including alcohol, specific gravity at 20°C, dry extract, reducing sugars, total and volatile acidity, pH, free and total SO_2. The results showed that variety significantly affected the chemical composition of wines, presenting higher contents of alcohol, density and dry extract for Vranec wines, while Smederevka wines were richer with total acids and contained higher amount of total SO_2. In addition, slight influence of vintage was noticed for both varieties, especially on the alcohol, specific gravity and dry extract contents, for which highest values were noticed in vintage 2017 in Vranec wines, while for Smederevka wines, values were highest in vintage 2020. Principal component analysis showed a clear separation of the wines according to the variety.

Key words: *variety, vintage, Smederevka, Vranec, basic physico-chemical parameters.*

INTRODUCTION

Wine production in the Republic of N. Macedonia has a long and significant history. Macedonian wines are characterized by intense aromas that are the result of the combined influence of two climates, mediterranean and continental. According to the climatic characteristics and classification of the European Union, the Republic of N. Macedonia is categorized as III-C-b zone for growing vines. In Macedonia, the wine-growing region is divided into three regions, as follows (Official Gazette of the Republic of Macedonia, No. 12, 1980; Official Gazette of the Republic of Macedonia, No. 74, 2024):

1) Povardarie Region (Vardar valley or Central Wine Region), which includes about 87% of the total grape production in the country;
2) Pelagonija-Polog Region (Western Region), which covers about 7% of the total grape production in the country, and
3) Pčinja-Osogovo Region (Eastern region), which covers about 6% of the total grape production in the country.

These three regions are divided into 16 sub-regions (vineyards) characterized by different production conditions and production intensity.

Wine production is a complex technological process which includes several procedures, such as: harvesting the grapes, crushing the grapes (separating the stems from the berries), adding SO_2, pressing, fermentation, maceration (mostly applied to red wines), clarification, stabilization, ripening and wine bottling. Determining the right time to harvest grapes, the structure, grapes condition and quality is performed based on the appearance, taste and analyses (sugar content, acidity, total polyphenols) of the

grapes and bunches. In order to preserve the freshness, fruity aroma and taste of the grapes, it is best to harvest early in the morning, when the temperatures are low.

After *addition of* SO_2, the grape mash is inoculated with yeast (*Saccaromyces cerevisie*) to start the *alcoholic fermentation*, during which the carbohydrates are converted into ethyl alcohol and carbon dioxide, releasing energy. The choice of the type of yeast depends on the grape variety as well as the characteristics of the wine that the producer wants to obtain (Lin et al. 2012). During the alcoholic fermentation, suitable mineralized nutrients necessary for the metabolism of the yeast are added into the mash in order to support the reproduction of the yeast (mostly in the form of diammonium phosphate and nitrogen). The alcoholic fermentation is a complex biochemical process which depends on many factors such as: temperature, content of sugars, pH, acidity, presence of phenolic compounds as well as the content of the produced alcohol (Fleet, 2003, Divol et al., 2012, Mamolar-Domenech et al. 2023, Vion et al. 2023).

Maceration is the period of contact between the solid parts of the grape (skins and seeds) and the grape juice. During maceration, the grape components are released, and between them, the phenolic compounds, including anthocyanins (in red grapes), phenolic acids, flavonoids and flavan-3-ols are the most important components for the wine quality, responsible for the colour and for the structure of the wine (Ivanova et al. 2011a; Ivanova et al. 2011b; Ivanova-Petropulos et al., 2014; Raičević et al. 2017). The quantities and ratios of these compounds is variable, depending on the composition of the grapes and their maturity. According to the grapes' quality, their maturity, the origin, as well as the type of wine that will be produced, the development of the maceration processes can be described as: short, long, carbonic, thermal, pre-fermentation cold and long maceration combined with fermentation. After finishing of the alcoholic fermentation, the second microbiological transformation or malolactic fermentation takes place in the wines. During malolactic fermentation in a presence of lactic acid bacteria, the content of malic acid decreases due to its conversion to lactic acid, which concentration increases in the wine.

The next step after the fermentation, is *racking off* the wine, which removes the coarse particles that can lead to cloudiness. The first racking off is carried out after the end of the quiet fermentation, usually in November or December, while the second racking off is carried out in February or March, with the previous addition of sulphur dioxide, as an additional protection against oxidation. *Fining* of the wines is performed by substances (fining agents) in order to bind suspended particles into larger molecules that precipitate out more rapidly. The common fining agents include (a) organic agents: egg whites, casein (milk), gelatine and isinglass (fish bladders) and (b) mineral materials: bentonite clay, activated carbon, silica and kaolin. Before *bottling*, for stabilization, wines are *filtered* in order to eliminate microbiological phenomena caused by residual active yeasts and lactic acid bacteria. Also, wine aging is necessary to be performed in a period of few days (for young wine, e.g. Beaujolais wine) to nine months, eighteen months or even longer period of time, depending of the wine style and quality that is expected to be obtained. Maturation is usually performed in stainless steel tanks, concrete tanks or oak tanks.

Since the wine quality depends on climate conditions of the years, the vintage year has a statistically significant influence on the all parameters in wine (Hosu et al. 2016, Raičević, et al. 2017, Jovanović-Cvetković et al. 2023). The aim of this study was to examine the effect of variety and vintage on the basic physico-chemical composition of Smederevka and Vranec wines (the dominant white and red grape varieties in Republic of N. Macedonia, with the highest impact on the wine production and financial benefits of the country), produced in six consecutive years, produced from the grapes grown in Ovce Pole sub-region, which is also important wine subregion, but not very well studied. Therefore, the results will ensure valuable data for the influence of the variety and vintage, especially focused on one su-bregion, important for the vine growers and wine producers from Ovce Pole and from the whole country, in general.

MATERIAL AND METHODS

Chemicals and reagents

For performing the chemical analysis, the following chemicals and reagents, with analytical grade of purity, have been used: NaOH, concentrated H_2SO_4, $Na_2S_2O_3$, phenolphthalein, bromothymol blue, starch, KI, Feling I, Feling II and buffers (pH: 4, 7 and 7), all of them purchased from Alkaloid (Skopje). Ultra-pure deionized water with 0.0005 μS conductivity was obtained with a membrane filtration unit (Millipore, Molsheim France).

Winemaking

White winemaking. Grapes from Smederevka (*Vitis vinifera* L.) variety grown in Ovce Pole sub-region, have been harvested at optimal technological maturity (average value: 19.8° Brix) and used for wine production (vintages 2018-2023). Ovce Pole wine sub-region is located around the cities of Sveti Nikole, Stip and Probistip, north from the river Zletovo and covers area of 2456 hectares vineyards, at attitude of 300 to 560 m.

Harvested grapes (about 1 million kg) were transported to Vineks winery (with capacity of 1.8 million litres, located in Sveti Nikole, Ovce Pole wine sub-region), for wine production. Grapes were processed using electrical inox crusher/ destemmer, and the grape juice was immediately separated from the pomace and placed in a tank. Then, SO_2 was added into the grape juice (ca. 10 g/100L), followed with inoculation with *Saccharomyces cerevisiae* yeast strain (Fermactive Blanc Aromatique, Sodinal, Bulgaria, in a dose of 20 g/100 L) and addition of balanced nutrients composed of organic nitrogen, mineral nitrogen and vitamins (Fermactive Activateur Complexe, Sodina, Bulgaria, in a dose of 10 g/100 L) for yeast nutrition. In addition, enzyme (Speed up Blanc, Sodinal, Bulgaria, in a dose of 10 g/100L) and tannin (Tanivin Blanc, Sodinal, Bulgaria, in a dose of of 10 g/100L) were added into the juice in order to ensure fast and efficient clarification and stabilization of redox potential of wine. After finishing the alcoholic fermentation, wine was stabilized with bentonite (2 g/L) and followed with addition of a product consisting of metatartaric acid and gum Arabic (10 g/100 L) (MetaGum, Erbslöh, Geisenheim, Germany) to ensure long-term crystal wine stabilization.

After 5-6 months of storage, wine was bottled. Before bottling, wine was three times filtered, at first passing thought a filter with pore sizes of 1.3 microns, followed with passing of the wine thought a filter with pore sizes of 0.9 microns and 0.6 microns and additionally protected with appropriate amount of SO_2. A total of 700 000 litres of Smederevka wine was produced.

Red winemaking. Grapes from Vranec (*Vitis vinifera* L.) variety grown in Ovce Pole wine sub-region, have been harvested at optimal technological maturity (average value: 22.9° Brix) (vintages 2017-2022). Harvested grapes (about 1.5 million kilograms) were transported to Vineks winery for wine production. Grapes were processed using electrical inox crusher/ destemmer, then added with SO_2 (ca. 10 g/100L) before inoculation with *Saccharomyces cerevisiae* yeast strain (Fermactive aroma varietale, Sodinal, Bulgaria, in a dose of 20 g/100 L), followed with addition of nutrients (Fermactive Activateur Complexe, Sodina, Bulgaria, in a dose of 10g/100 L) for the yeast nutrition. The grape mash was macerated for 5-6 days at 25-30 °C, with pumping over and delastage performed twice per day, followed by separation of the wine from the sediment and treatment with bentonite (1 g/L) and with a product consisting of metatartaric acid and gum Arabic (10 g/100 L) (MetaGum, Erbslöh, Geisenheim, Germany) to ensure long-term crystal stabilization in wine. After 5-6 months of storage, wine was bottled. Before bottling, wine was two times filtered, at first passing thought a filter with pore sizes of 1.3 microns, followed with passing of the wine thought a filter with pore sizes of 0.9 microns and additionally protected from oxidation by adding appropriate amount of SO_2. A total of 1 million litres of Vranec wine was produced.

Principal chemical composition

The principal chemical composition of wines was determined using the official methods of analysis of wines (OIV 2022). The following parameters have been determined: alcohol (OIVMA-AS312-01 A), dry extract (OIV-MA-AS2-03B), specific density (OIV-MA-AS2-01 A), total acidity (OIV-MAAS313–01), volatile acidity (OIV-MA-AS313–02), pH (OIV-MA-AS313-15), reducing sugars, free SO_2 and total SO_2 (Ivanova-Petropulos and Mitrev 2014).

Statistical analysis

Each wine was analysed in three replicates. Results were statistically treated including determination of means, minimum, maximum, standard deviation (SD) and relative standard deviation (RSD), calculated using Microsoft Excel (2013). Principal component analysis was performed on the results for both wine varieties produced in 6 various years, using the XLStat software (Addinsoft, Version 2015.5.01.22537), in order to study possible groupings of wines as an influence of the vintage and variety.

RESULTS AND DISCUSSION

The basic physico-chemical parameters that are important for wine quality are: alcohol, dry extract, specific density, total acidity, volatile acidity, pH, reducing sugars, free SO2 and total SO2. These parameters were determined for wine samples from Smederevka and Vranec varieties produced during 6 consecutive years (period of 2018 to 2023 for Smederevka and 2017 to 2022 for Vranec). The results for the basic parameters of the wines are shown in Tables 1 and 2.

Influence of variety

The alcohol content, which is considered as one of the most important factors for wine quality and stability, ranged from 10.7 to 11.26% in the wine samples from both varieties in accordance to the literature data (Neceva et al. 2016). Vranec wines contained slightly higher content of alcohol (on average: 11.52%) compared to Smederevka wines (on average: 10.8%), regardless of the year of production, probably because of the higher content of reducing sugars in Vranec grapes compared to Smederevka grapes.

The values for specific gravity in Smederevka wines ranged from 0.9913 to 0.9944 (on average: 0.9931), which were lower than the specific gravity values of Vranec, ranged from 0.9936 to 0.9961 (on average: 0.9953). The obtained results were expected and comparable to the specific gravity for white and red wines published in the literature, which usually range from 0.9912 to 1.0038 (Piperevski, et al. 2023; Budziak-Wieczorek et al., 2023). In fact, the specific gravity is important parameter that is necessary to determine the dry extract of wine. Wine contains a mixture of dissolved nonvolatile solids, such as carbohydrates, acids, phenols, glycerol etc., which increase the specific gravity. Since red wine usually contains higher content of phenols, especially anthocyanins which are not present in white wine, it is expected red wines to have higher density compared to white wines.

In accordance to the specific gravity and in accordance to the literature (Ivanova-Petropulos et al., 2015, Pajović-Šćepanović et al., 2016, Piperevski et al., 2023), the wine dry extract values were lower in white wines, compared to red wines (regardless the vintage), ranged from 20.8 g/L (on average for Smederevka wines) to 28.3 g/L (on average for Vranec wines). Higher values of dry extract mean higher amount of extractive components from the grapes, fuller and stronger wine. Moreover, the values for density in the Macedonian Vranec wines examined in this study, were much higher compared to the Montenegrin Vranec (Vranac) wines (Pajović-Šćepanović et al., 2016). Since the climate conditions, such as temperature, influence on the grape and wine composition and quality, the higher temperatures in Macedonia and different geographic region are the main factors influencing on higher non-volatile compounds, such as polyphenols in Vranec grapes, and then in the corresponding wines, resulting with higher density of the wines.

Table 1. Basic physic-chemical composition of Smederevka wines produced in various vintages.

Vintage/ Parameters	Specific gravity at 20°C	Alcohol (%, *v/v*)	Total dry extract (g/L)	Total acidity (g/L)	Volatile acidity (g/L)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	pH	Reducing sugars (g/L)
2018	0.9939	10.7	21.3	5.6	0.56	23.1	75.5	3.53	1.0
2019	0.9919	10.76	18.3	5.3	0.57	22.3	62.0	3.18	1.6
2020	0.9944	10.8	23.7	5.8	0.55	24.3	71.7	3.21	1.9
2021	0.9913	11	19.1	7.2	0.3	18.6	70.9	3.19	1.3
2022	0.9938	10.86	21.6	4.8	0.31	16.4	68.4	3.17	2.2
2023	0.9928	10.21	17	6.2	0.53	23.2	63.4	3.33	0.6
Average	**0.99306**	**10.8**	**20.8**	**5.74**	**0.46**	**20.9**	**69.7**	**3.25**	**1.6**
Min	**0.9913**	**10.7**	**18.3**	**4.8**	**0.3**	**16.4**	**62.0**	**3.17**	**1.0**
Max	**0.9944**	**11**	**23.7**	**7.2**	**0.57**	**24.3**	**75.5**	**3.53**	**2.2**
SD	**0.001**	**0.11**	**2.15**	**0.90**	**0.14**	**3.31**	**5.00**	**0.15**	**0.47**
RSD (%)	**0.14**	**1.06**	**10.3**	**15.7**	**30.5**	**15.8**	**7.18**	**4.73**	**29.6**

Abbreviations: Min - minimum; Max - maximum; SD - standard deviation; RSD - relative standard deviation.

Table 2. Basic physic-chemical composition of Vranec wines produced in various vintages.

Vintage/ Parameters	Specific gravity at 20°C	Alcohol (%, *v/v*)	Total dry extract (g/L)	Total acidity (g/L)	Volatile acidity (g/L)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	pH	Reducing sugars (g/L)
2017	0.9961	11.62	29.8	5.4	0.68	16.6	44.8	3.71	1.0
2018	0.9953	11.38	27.0	5.5	0.71	23.1	43.5	3.51	1.3
2019	0.9959	11.42	28.8	5.7	0.49	19.2	33.3	3.40	2.2
2020	0.9955	11.56	28.2	4.6	0.48	29.4	39.4	3.53	1.0
2021	0.9936	11.61	27.8	5.0	0.42	23.2	40.4	3.45	1.9
2022	0.9955	11.53	28.0	4.6	0.70	19.4	44.8	3.50	1.9
Average	**0.9953**	**11.52**	**28.3**	**5.13**	**0.58**	**21.8**	**41.0**	**3.51**	**1.6**
Min	**0.9936**	**11.38**	**27.0**	**4.6**	**0.42**	**16.6**	**33.3**	**3.40**	**1.0**
Max	**0.9961**	**11.62**	**29.8**	**5.7**	**0.71**	**29.4**	**44.8**	**3.71**	**2.2**
SD	**0.001**	**0.10**	**0.95**	**0.47**	**0.13**	**4.49**	**4.41**	**0.11**	**0.52**
RSD (%)	**0.09**	**0.86**	**3.37**	**9.19**	**22.5**	**20.6**	**10.7**	**3.01**	**33.3**

Abbreviations: Min - minimum; Max - maximum; SD - standard deviation; RSD - relative standard deviation.

In addition, the concentration of reducing sugars, composed by the main carbohydrates in grapes, glucose and fructose, was very low in the analysed wines from both varieties. The content ranged from 1 to 2.2 g/L in both wines. All wines were dry (< 5 g/L) and in all of them the fermentation ended successfully, which was in accordance to the previous published data (Piperevski et al., 2023).

Total wine acidity is a sum of non-volatile and volatile acidity, and includes all types of acids, such as formic acid, organic acids (tartaric, malic and citric), as well as amino acids. The total acidity is expressed in tartaric acid equivalents since the tartaric acid is the predominant acid in must and wine (Piperevski et al., 2023). In this study, the average concentration of total acidity in Smederevka wines was 5.74 g/L, which was slightly higher compared to acidity in Vranec wines (5.13 g/L). These values are relatively high and sufficient to ensure satisfactory chemical and microbiological stability of wines as well as sufficiently optimal freshness. Red wines are stable even at lower acidity due to the presence of phenols that enable stability of the wines (Piperevski et al., 2023).

pH is another factor that influence the wine stability and freshness. In this study, the average pH values were: 3.25 for Smederevka wines and 3.51 for Vranec wines, which was in accordance to the total acidity in the wines (higher total acidity leads to lower pH values). In fact, the values in range between 3.14 to 3.6 are considered as normal and typical values for wines (Piperevski et al., 2023).

The volatile acidity showed an overall average value of 0.46 g/L for Smederevka wines and 0.58 g/L for Vranec wines with no influence

on the quality of wines that were protected from further oxidation and microbial contamination by the free SO_2 present in a sufficient level in the wines (16.4 to 29.4 mg/L) and sufficient total SO_2 (41 to 69.7 mg/L) (Ivanova-Petropulos et al., 2015). According to the stated regulations, the maximum allowed content of volatile acidity is 1.2 g/L acetic acid for red wines (Official Gazette of the Republic of Macedonia, No 16, 2012).

Influence of vintage

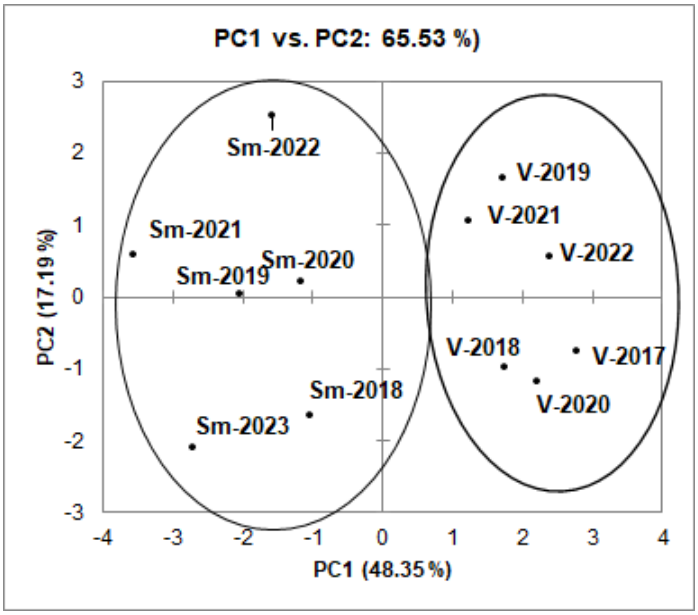
By observing the influence of the vintage during six consecutive years, slight differences have been noticed in both varieties. The main parameters influenced by the vintages have been alcohol, specific gravity and total dry extract, in accordance to the literature (Raičević et al., 2017). Free SO_2, total SO_2 and volatile acidity are factors that mainly are not influenced by the vintage and no significant differences have been noticed between wines from both varieties. Thus, the highest values of alcohol, specific gravity and total dry extracts were noticed in vintage 2020 for Vranec wines and in vintage 2017 for Smederevka wines. The contents of reducing sugars were very similar between all wines meaning that the conversion of glucose and fructose during the alcoholic fermentation was completed in all wines. Since we do not have data for the air temperature and precipitation, we cannot make any specific conclusion about influence of climate conditions of the grape composition.

Principal component analysis (PCA)

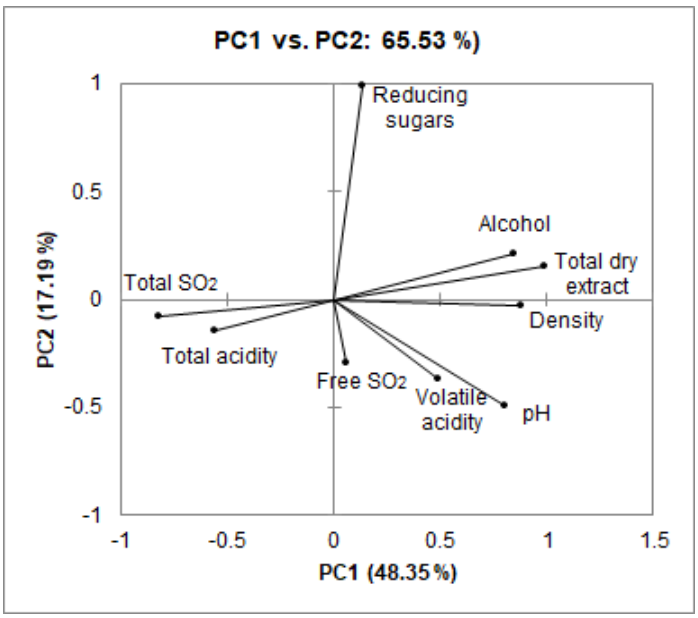
Principal component analysis (PCA) was applied to explore the contribution of each physico-chemical parameter on possible grouping among the analysed wines. PCA was performed in order to evaluate the effect of each factor (variety and vintage) on the physico-chemical profile of the analyzed Smederevka and Vranec wines and to identify the parameters that best discriminate the wines. The first two principal components, PC1 and PC2, accounted for 65.53% of the total variance (48.35% for PC1 and 17.19% for PC2). Projection of the wines on the first two principal components showed a separation mainly according to the variety (Fig. 1a). Thus, the wines from Smederevka variety, located at the negative part of PC1, were clearly separated from the wines from Vranec variety, located in the positive part of PC1. Further, wines were grouped and divided into subgroups according to the vintage. Thus, Vranec wines from vintages 2017, 2018 and 2002 were located at the positive part of PC2, while Vranec wines from 2019, 2021 and 2022 vintage were separated and located in the positive part of PC2. Concerning Smederevka variety, wines from vintages 2018 and 2023 were located in the negative part of PC2, while all other wines (2019-2022) were grouped in the positive part of PC2. In this respect, PC1 was mostly related to the variety, while PC2 to the vintage.

PCA results of the variables used for physic-chemical characterization of the wine samples displayed into the first two principal components are presented in the scatter plot in Fig. 1b. It could be noticed that only total acidity and total SO_2 prevail in the negative part of PC1, while all other parameters prevailed in the positive part of PC1. In fact, total acidity and total SO_2 were discriminant factors for the white wines and their content was higher in Smederevka wines, since white wines have to be protected with higher dose of SO_2 due to the absence of anthocyanins and lower dose of phenolic compounds in general, compared to red wines. For better stability, higher acidity in white wines is typical.

In general, the stronger correlation between alcohol, density and dry extract was notices, since all these parameters depends on each other and are considered as very important factors defining the wine quality in general.



(a)



(b)

Fig. 1. Principal component score plot (a) and correlation scatterplots (b) of the variables with PC1 and PC2 based on physico-chemical parameters for Smderevka and Vranec wines produced in six consecutive vintages.

CONCLUDING REMARKS

Vranec and Smerevska wines (*V. Vinifera* L. *cv*.) have been produced in 6 (six) consecutive years in order to study the influence of variety and vintage on the general physico-chemical parameters. It was noticed that variety significantly affected the chemical composition of wines, presenting higher contents of alcohol, specific gravity and dry extract for Vranec wines, while Smederevka wines contained higher concentration of total acids and higher amount of total SO_2. Influence of vintage was noticed for both varieties, observing highest values of alcohol, specific gravity and dry extract in vintages 2017 and 2020 for Vranec and Smederevka wines, respectively. Principal component analysis presented grouping of the wines mainly according to the variety.

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ВЛИЈАНИЕ НА СОРТАТА И ГОДИНАТА НА БЕРБА ВРЗ ОСНОВНИОТ ФИЗИЧКО- ВЛИЈАНИЕ НА СОРТАТА И ГОДИНАТА НА БЕРБА ВРЗ ОСНОВНИОТ ФИЗИЧКО-ХЕМИСКИ СОСТАВ НА ВИНА ОД СОРТИТЕ СМЕДЕРЕВКА И ВРАНЕЦ

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Резиме

Во ова истражување произведени се вина од сортите Смедеревка и Вранец (*V. Vinifera* L. *cv*.) во текот на шест последователни години, со примена на традиционални ферментациони методи, со цел да се проучи влијанието на сортата и годината на берба врз основниот квалитет на вината. Беа определени физичко-хемиски параметри со кои се потврдува основниот квалитет на виното, вклучувајќи алкохол, специфична тежина на 20°C, редуцирачки шеќери, вкупна и испарлива киселост, pH, слободен и вкупен SO_2. Резултатите покажаа дека сортата значително влијае на хемискиот состав на вината, при што највисоки содржини на алкохол, специфична тежина и вкупен сув екстракт се забележани за вината од сортата Вранец, додека вината од сортата Смедеревка содржеа најмногу вкупни киселини и вкупен SO_2. Дополнително, беше забележано мало влијание на годината на берба врз хемискиот состав на вината од двете сорти, особено на параметрите алкохол, специфична тежина и вкупен сув екстркат. Притоа, највисоки вредности за алкохол, специфична тежина и вкупен сув екстркат беа забележани во 2020 година за вината од сортата Вранец, додека за вината од сортата Смедеревка највисоки вредности на овие параметри се забележани во 2017 година. Анализата на главните компоненти покажа јасно сепарирање на вината според сортата.

Клучни зборови: *сорта, година на берба, Смедеревка, Вранец, основни физичко-хемиски параметри.*

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