Національний університет біоресурсів і природокористування України

БІОЛОГІЧНІ СИСТЕМИ: Теорія та Інновації

Том 16, № 4
2025

Київ 2025

ISSN 2706-8382
e-ISSN 2706-8390

Засновник:
Національний університет біоресурсів і природокористування України

Рік заснування: 2010

Рекомендовано до друку та поширення
через мережу Інтернет Вченою радою
Національного університету біоресурсів і природокористування України
(протокол № 5 від 27 листопада 2025 р.)

Державна реєстрація: Ідентифікатор медіа R30-02292.
Рішення Національної Ради України
з питань телебачення і радіомовлення
№ 1795, протокол № 31 від 21.12.2023 р.

Журнал включено до категорії «Б» Переліку наукових фахових видань України,
відповідно до якого можуть публікуватися результати дисертаційних робіт на здобуття наукових ступенів доктора та кандидата біологічних наук зі спеціальностей
(наказ Міністерства освіти і науки України № 409 від 6 березня 2020 року):
091 – Біологія; 101 – Екологія; 162 – Біотехнології та біоінженерія; 202 – Захист і карантин рослин

Журнал представлено у міжнародних наукометричних базах даних, репозитаріях та пошукових системах:
Open Ukrainian Citation Index, University of Hull Library, University of Oslo Library, WorldCat, Crossref, Національна бібліотека України імені В.І. Вернадського, BASE, EBSCO, Google Академія, Ulrichsweb Global Serials Directory, UCSB Library, Dimensions, Litmaps, AGRIS

Адреса редакції:
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 13, м. Київ, Україна
E-mail: bs@bioscience.com.ua
https://bioscience.com.ua/uk

National University of Life and Environmental Sciences of Ukraine

BIOLOGICAL SYSTEMS: Theory and Innovation

Vol. 16, No. 4
2025

Kyiv 2025

ISSN 2706-8382
e-ISSN 2706-8390

Founder:
National University of Life and Environmental Sciences of Ukraine

Year of foundation: 2010

Recommended for printing and distribution
via the Internet by the Academic Council
of National University of Life and Environmental Sciences of Ukraine
(Minutes No. 5 of November 27, 2025)

State registration: Media identifier R30-02292.
Decision of the National Council of Television
and Radio Broadcasting of Ukraine
No. 1795, Minutes No. 31, dated 21.12.2023.

The journal is included in category "B" of the List of scientific professional publications of Ukraine, in which the results of dissertations for the degree of doctor and candidate of biological sciences can be published in the following specialties
(Order of the Ministry of Education and Science of Ukraine No. 409 dated 06.03.2020):
0511 – Biology; 0521 – Environmental sciences; 0588 – Interdisciplinary programmes and qualifications involving natural sciences, mathematics and statistics; 0712 – Environmental protection technology

The journal is presented in the international scientometric databases, repositories and scientific systems:
Open Ukrainian Citation Index, University of Hull Library, University of Oslo Library, WorldCat, Crossref, Vernadsky National Library of Ukraine, BASE, EBSCO, Google Scholar, Ulrichsweb Global Serials Directory, UCSB Library, Dimensions, Litmaps, AGRIS

Editors office address:
National University of Life and Environmental Sciences of Ukraine
03041, 13 Heroiv Oborony Str., Kyiv, Ukraine
E-mail: bs@bioscience.com.ua
https://bioscience.com.ua/en

Редакційна колегія

Editorial Board

Зміст/Contents

Received: 02.07.2025 Revised: 23.10.2025 Accepted: 27.11.2025 Published: 26.12.2025

BIOLOGICAL SYSTEMS: Theory and Innovation

ISSN 2706-8382
e-ISSN 2706-8390

Journal homepage: https://bioscience.com.ua/en
Vol. 16, No. 4, 2025, 10-22

UDC [528.01/.02:574:502/504.064.3](477.86–751.2/.3) DOI: 10.31548/biologiya/4.2025.10

GIS analysis of the boundaries of the Carpathian National Nature Park: Comparison of global and local geodata sources and their importance for environmental monitoring

Petro Nasadiuk*
PhD Student
Ivan Franko National University of Lviv
79000, 1 Universytetska Str., Lviv, Ukraine
https://orcid.org/0009-0008-8189-4945

Zvenyslava Mamchur
PhD in Biological Sciences, Associate Professor
Ivan Franko National University of Lviv
79000, 1 Universytetska Str., Lviv, Ukraine
https://orcid.org/0000-0003-0527-5639

Oksana Tymchuk
Scientist
Carpathian National Nature Park
78500, 6 Vasyl Stus Str., Yaremche, Ukraine
https://orcid.org/0009-0000-4728-9401

Abstract. The relevance of the study lies in the need to ensure high accuracy in determining the boundaries of the Carpathian National Nature Park (CNNP), which is important for the reliability of environmental monitoring using geographic information systems (GIS) and field studies. The conservation of natural ecosystems and effective management of the Carpathian National Nature Park territory are impossible without the integration and analysis of spatial data in geographic information systems, which ensure sound planning of nature conservation measures and control of the state of the environment. The aim of the article was to analyse geospatial datasets (boundary polygons) of the CNNP available in open sources and official materials, compare their accuracy and suitability for analysing environmental indicators, conduct spot field verification, and develop the authors' version of the park's refined boundaries. The study analysed the possibilities of using open geospatial data, in particular satellite images, digital terrain models, and thematic layers of the Global Forest Watch platform in QGIS and ArcGIS environments. A comparison of different sources revealed significant errors in publicly available datasets, leading to distortions in statistical estimates of forest area, land use changes, and the identification of areas of anthropogenic impact. Based on geospatial analysis, field surveys, and comparison of official materials, the authors propose their own version of the refined boundaries of the CNNP. The paper provides an example of how inaccuracies in the boundaries of global monitoring systems, in particular on the Global Forest Watch platform, can significantly alter estimates of deforestation or forest degradation areas. This highlights the need for constant verification and updating of geospatial data for protected areas. The results of the study prove that the combination of

Suggested Citation:
Nasadiuk, P., Mamchur, Z., & Tymchuk, O. (2025). GIS analysis of the boundaries of the Carpathian National Nature Park: Comparison of global and local geodata sources and their importance for environmental monitoring. *Biological Systems: Theory and Innovation*, 16(4), 10-22. doi: 10.31548/biologiya/4.2025.10.

*Corresponding author

open data, field research and modern GIS technologies is an effective tool for improving the accuracy of environmental data analysis. The proposed approach can be used as a model for improving the spatial boundaries of other protected areas in Ukraine, promoting sustainable nature use and transparent ecosystem management

Keywords: open data; geospatial layer; satellite imagery; QGI; ArcGIS

INTRODUCTION

Accurate spatial and temporal characterisation of the boundaries of nature conservation areas determines the quality of environmental monitoring, planning of conservation measures and reporting on international environmental obligations. For the Carpathian National Nature Park, located in a complex mountain system with high landscape and biotic diversity, errors in contours can cause systematic deviations in ecosystem area calculations, anthropogenic impact assessments, and site selection for observations (Urbano *et al*., 2024). In the context of multi-source mapping (global databases, national registers, local geodata), it is necessary to verify the geospatial consistency and methods of integrating such data in order to obtain a single reliable boundary model suitable for management and scientific tasks.

The Carpathian National Nature Park (CNNP) is one of the most important nature conservation sites in Ukraine, located within the Chornohora, Gorgany, and the Pokuttya-Bukovyna Carpathians. Administratively, the park covers the Yaremche, Vorokhta, and Polyanytsya territorial communities of Ivano-Frankivsk region. Its official area is 50,495 hectares, and it encompasses unique mountain ecosystems, including subalpine meadows, beech and fir forests, high-altitude bogs, and rare geological formations (Carpathian National Nature Park, n.d.). It is home to a significant number of endemic, relict and red-listed species of flora and fauna, making the park critically important for biodiversity conservation. Due to the complex terrain, high ecological value and limited area, the accuracy of geospatial data is critical for environmental analysis, nature conservation planning and international reporting.

In scientific works, the issues of boundary compliance, geodata quality and methods of their comparison for protected areas are considered from different perspectives. V. Brusak & V. Shtuglynets (2021) demonstrated the potential of GIS mapping for the systematic inventory of the nature reserve fund (NRF) of the Ukrainian Carpathians: QGIS was used to compile maps of the location of NRF objects by landscape complexes and to assess the representativeness of landscape types; the authors recommended optimising the network of parks for a balanced representation of mountain landscape types. A. Novikov (2021) developed GIS maps of the geomorphological and phytogeographical zoning of the Ukrainian Carpathians in the form of operational geographical units, which increases the consistency of local taxa with modern digital sets and ensures routine use in biogeography. L.V. Shtohryn & D.V. Kasiynchuk (2024), based on morphometric analysis and multivariate statistics, showed the dominance of orographic and climatic factors in the formation of landslide processes in the Carpathian region; the consistency of the model parameters reinforces the requirement for high spatial accuracy of the initial boundaries during territorial hazard assessments. The authors R.L. Kravchynskyi *et al*. (2025) systematised the abiotic components of the environment and described field research and monitoring methods, emphasising the need for validated boundaries as a framework for thematic studies.

The study by J. Randall *et al.* (2022) shows that the integration of field research and interactive visualisations in GIS for invasive species management: mapping spatio-temporal patterns of distribution, creating web applications for navigation and queries, and visualising data from the field helps to align management tactics with landscape changes and prioritise areas of intervention. This approach reinforces the argument for the need for accurate and consistent park boundaries as a basis for spatial analysis and management planning. At the global source level, the Protected Planet/WDPA initiative provides monthly updates and standardisation of data on protected areas; at the same time, the WDPA User Guide outlines limitations in data interpretation and cases of point representation of objects, which affects local analyses. D. Tezel *et al*. (2021) demonstrated the use of 3D GIS and high-resolution datasets to

accurately determine the boundaries of protected areas and align them with property boundaries, which is relevant for parks with complex internal land structures. The aim of the study was to compare global and local geodata sources regarding the boundaries of the Carpathian National Nature Park, to quantitatively assess their spatial consistency and errors, and to propose a methodology for constructing a verified GIS model of boundaries suitable for environmental monitoring of the park.

MATERIALS AND METHODS

The study was conducted at the Department of Ecology of Ivan Franko National University of Lviv during the summer of 2025. The following geospatial data sources were used in the study: The World Database on Protected Areas (WDPA) (n.d.) via Living Atlas in Pro – two polygons with an area of 612 km^2 and 500 km^2; OpenStreetMap (OSM) (n.d.) – the primary polygon, with an area of 529 km^2, exported from the platform, partially overlapped the territory of the Carpathian Biosphere Reserve and extended into the Zakarpattia region. This configuration of boundaries did not correspond to the administrative location of the CNNP, which is entirely within the Ivano-Frankivsk region. The author edited the polygon in accordance with the official boundaries of the region and the actual spatial coverage of the park. As a result, the area of the edited layer was 512 km^2, which more accurately reflects the actual territory of the CNNP. Official data from the Carpathian National Nature Park administration (2024) (unpublished materials from the park administration obtained by the authors upon request) was used – a polygon with an area of 499 km^2, which became the basis for further editing. All boundary changes were made exclusively on the basis of this original layer. It should be noted that all polygons with the boundaries of the CNNP analysed in this study were probably created as part of various projects, in particular OpenStreetMap (n.d.), The World Database on Protected Areas (n.d.), as well as an internal polygon used by the park administration for its own mapping, and none of them are officially approved.

This creates additional challenges for researchers using open sources for environmental analysis, as they may use CNNP boundaries from different sources, leading to significantly different characteristics and conclusions about a given territory. It should be noted that different datasets in open environmental information databases have differences in coding systems and cartographic projections, which often leads to incorrect calculations of the actual area of the territory. In most cases, such data only allow working with relative indicators expressed as percentages, rather than absolute values of areas.

To assess the accuracy of the boundaries, the following was carried out: visual analysis of Sentinel-2 satellite images (resolution 10 m) (European Space Agency, n.d.); comparison of boundaries with ecological trails, topographical landmarks and field observations; field verification of the inclusion of important natural features, such as the arboretum, the banks of the Prut River – the Hoverla Research and Conservation Scientific Department, and the peaks of the Chornohora Range. To ensure maximum accuracy of spatial analysis, all layers, including the base layer, were reprojected into the ETRS89 / UTM Zone 35N coordinate system, which is optimal for the park's location – for accurate area calculation. For analysis of the territory on Global Forest Watch (GFW), all polygons were reprojected into the WGS 1984 / EPSG 4326 coordinate system. Data from the Global Forest Watch platform (n.d.) was used to assess the importance of the accuracy of polygons reflecting the boundaries of the CNNP, as well as to demonstrate the practical impact of geometric deviations on environmental indicators.

Assessment of boundary consistency: "Deviation from intersection area (%)" indicator. The "Deviation from intersection area (%)" indicator was used to quantitatively assess the spatial consistency of different sources of park boundaries. Its purpose is to measure the relative size of the uncovered part of the compared polygon relative to the official (baseline) boundary in order to ensure the comparability of results between sets of different areas. A value of 0% means complete coincidence, while higher values indicate increasing discrepancies. In the ArcGIS Pro software environment, the Intersect function was used to create intersection layers for each of the polygons with the base polygon, and the attribute table data was used to determine the difference in the areas of the intersecting polygons, as well as the percentage of overlap with the base polygon. For further analysis

of territories by environmental indicators, it was important to understand how much the area intersecting with the base polygon differs from the area of the polygon itself. This indicator was calculated using the formula:

$$\text{Deviation of area from intersection} = \\ = |\text{Area of polygon} - \text{Area of intersection}| / \\ \text{Area of baseline polygon} \times 100. \quad (1)$$

Correlation analysis was performed in Microsoft Power BI (Desktop) on a sample of six polygons (Author, WDPA 612, WDPA 500, OSM 529, OSM 512, CNNP 499). Bubble scatter plots were constructed for pairs of variables: geometric/areal metrics and corresponding ecological indicators were plotted on the axes; the size of the markers is proportional to the polygon area, and the colour codes the data source. Data preparation included bringing percentage indicators to the baseline polygon (Authors') to ensure comparability between sources.

RESULTS AND DISCUSSION

The analysis revealed significant discrepancies between the geospatial data sources used to delineate the boundaries of the Carpathian National Nature Park. Although some layers show a high degree of spatial coincidence with the reference contour, even minor deviations in area and geometry can lead to significant distortions in the calculation of environmental indicators, such as forest loss, fire impact and forest cover growth. A visual comparison of the boundaries of all the geospatial layers considered is shown in Figure 1, which illustrates the nature and scale of spatial discrepancies.

The territory of the CNNP according to The World Database on Protected Areas (n.d.): WDPA 612 – its difference from the base area is 21.3%, the degree of territorial overlap is 86%, and the deviation of its area from the intersection is already 35%. This means that if the WDPA 612 polygon is used to analyse the territory of the CNNP, 35% of its area will not correspond to the actual territory of the CNNP. Thus, it should be emphasised that this geospatial layer cannot be used even for superficial generalised studies or schematic representations of the CNNP. On the other hand, the WDPA 500 polygon has a slight deviation in area – only -0.97%, but 5.8% of its territory is not within the boundaries of the CNNP, and the degree of spatial coincidence with the reference polygon is only 93.23%. The visualisation of these layers is shown in Figure 2.

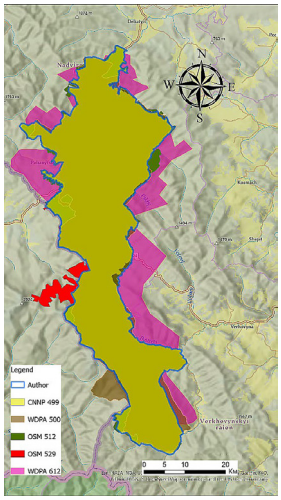

Figure 1. Spatial overlay of the boundaries of the authors' layer of the CNNP with the layers of the WDPA, OSM, and the official CNNP layer
Source: developed by the authors based on The World Database on Protected Areas (n.d.), OpenStreetMap (n.d.)

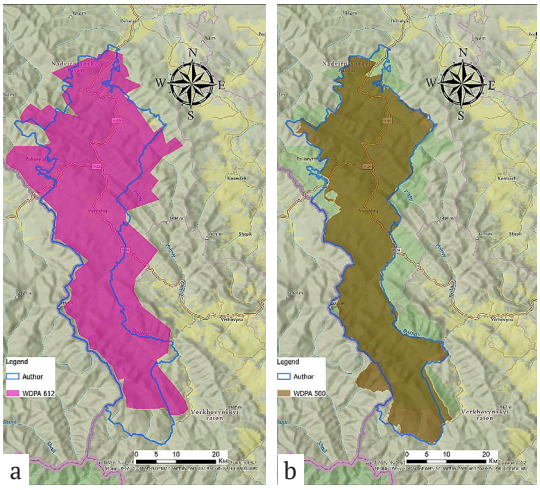

Figure 2. Spatial overlay of the Carpathian NNP boundaries with the authors' layer
Note: a – with the WDPA 612 layer; b – with the WDPA 500 layer
Source: developed by the authors based on The World Database on Protected Areas (n.d.)

CNNP according to OpenStreetMap (n.d.) (Fig. 3): OSM 529 (obtained by direct export to QGIS via the Quick OSM plugin) – the area of the polygon is 529.37 km^2, which is 4.84% larger than the reference area. The degree of territorial overlap with the authors' polygon is 98.60%, i.e. only 1.4% of its territory is not included in the CNNP. At the same time, the deviation of the area from the intersection is 6.24%, which indicates a moderate discrepancy in geometric coverage. It should be noted that this geospatial layer contains a gross error and includes only part of the Carpathian Biosphere Reserve (CBR) (n.d.), namely the Nature Conservation Bureau (n.d.), so it cannot be used as representative of the CNNP. OSM 512 has an area of 512.25 km^2, which is 1.45% less than the reference area. The degree of spatial overlap is 98.57%, meaning that 1.43% of the territory does not correspond to the boundaries of the CNNP. The deviation of the area from the intersection is 2.88%, which is minimal and indicates high accuracy. This polygon is the closest to the authors' among the OSM layers and can be recommended for use in environmental monitoring, but with caution regarding the edge areas.

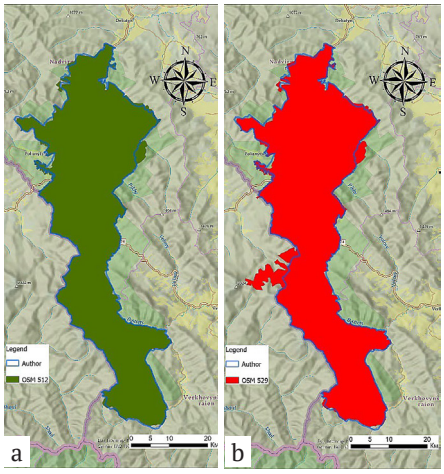

Figure 3. Spatial overlay of the Carpathian NNP boundaries with the authors' layer
Note: a – with OSM layer 512; b – with OSM layer 529
Source: developed by the authors based on OpenStreetMap (n.d.)

The territory of the Carpathian National Nature Park (n.d.) according to the geospatial layer of the CNNP 499: the most accurate, but does not include some ecologically important areas, the area of the polygon is 499.87 km^2, which is 1.01% less than the authors' polygon. The degree of territorial overlap is 98.92%, i.e. only 1.08% of its area does not correspond to the actual boundaries of the CNNP. The deviation of the area from the intersection is 0.08%, which is minimal and indicates high accuracy of geometric coverage. This polygon is the closest to the authors' among all the layers considered, both in terms of area and spatial coincidence (Fig. 4). It can be considered as a basis for use in environmental monitoring, spatial analysis and planning of nature conservation measures. As a result of the authors' reconstruction of the boundaries, an updated polygon with a GIS area of 50,495 ha was created, which fully corresponds to the official data. The reconstruction was carried out exclusively on the basis of the CNNP 499 polygon, to which the territories of the Hoverla Research and Conservation Scientific Department arboretum, the riparian zone of the Prut River, and the peaks of the Chornohora Range were added, which were not included in the original polygon but are actually part of the park's territory according to its functional use and nature conservation documentation.

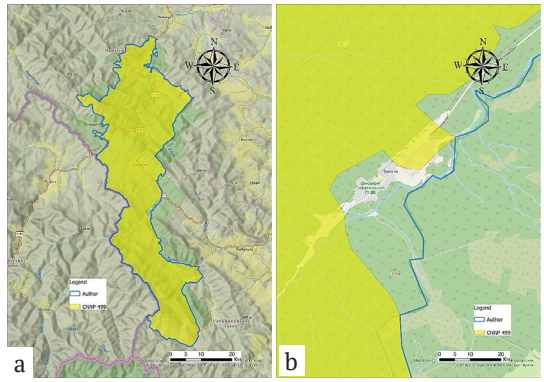

Figure 4. Spatial overlay of the Carpathian NNP boundaries with the authors' layer
Note: a – with the CNNP layer; b – preservation of the details of the authors' restoration on the territory of the arboretum
Source: developed by the authors

Table 1 shows the results of the analysis of polygon areas and their deviations, and Figure 5 shows a visualisation of correlations for various indicators. For comparative calculations and

further GIS analysis, the authors' version of the boundaries with an area of 504.95 km^2 was taken as the basis. As can be seen from Table 1, an important indicator is not only the difference in polygon areas, but also the degree of overlap, i.e. what part of the reference polygon coincides with the base polygon. In the ArcGIS Pro software environment, using the Intersect function, layers of intersection of each of the polygons with the base were created, and through the attribute table data, the difference in the areas of the polygons with the intersection was determined, as well as the percentage of overlap with the base polygon. For further analysis of the territories according to environmental indicators, it was important to understand how much the area intersecting with the base polygon differs from the area of the polygon itself (formula 1) – these data are given in the column Deviation from the intersection area (%) in Table 1.

Table 1. Comparison of CNNP polygons:
Contour overlap and geometric deviations from the authors' layer

Name of polygon	Polygon area (km^2)	Intersection area (km^2)	Percentage of overlap (%)	Difference between area and intersection (km^2)	Deviation of area from intersection (%)	Deviation from area (%)
Authors' 504	504.95	504.95	100.00	0.00	0.00	0.00
WDPA 612	612.57	435.24	86.19	177.33	35.10	21.31
OSM 529	529.37	497.87	98.60	31.50	6.24	4.84
OSM 512	512.25	497.72	98.57	14.53	2.88	1.45
WDPA 500	500.04	470.75	93.23	29.29	5.80	-0.97
CNNP 499	499.87	499.48	98.92	0.39	0.08	-1.01

Source: developed by the authors

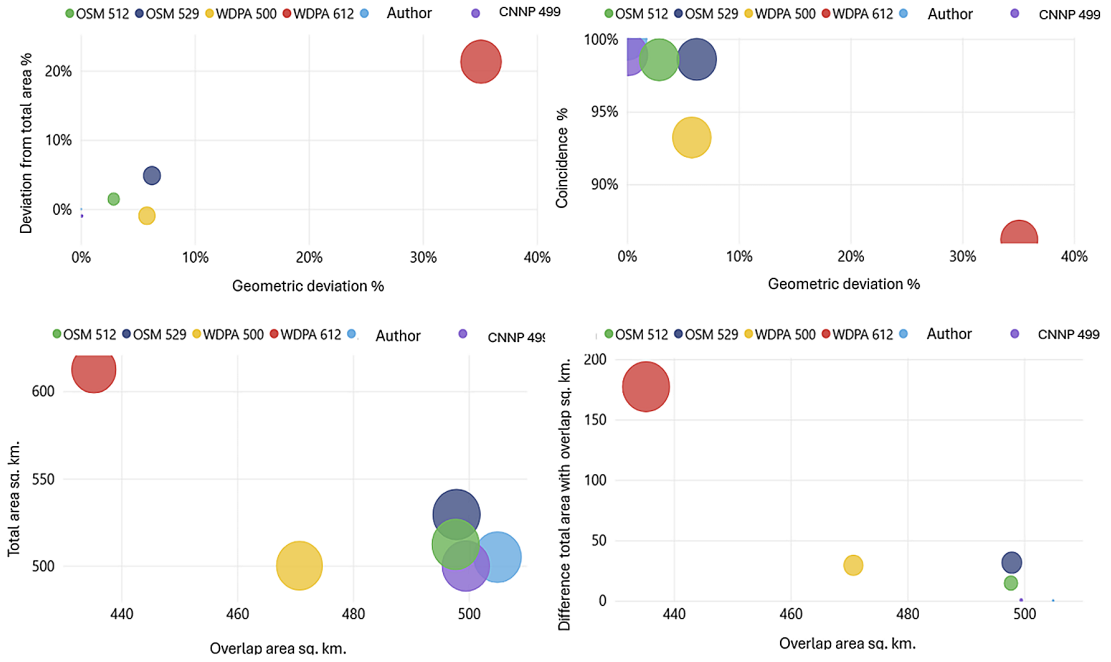

Figure 5. Correlation analysis of spatial characteristics of nature conservation areas boundaries: Relationships between geometric deviation, planar discrepancies and degree of territorial overlap
Source: developed by the authors

Global Forest Watch (n.d.) was selected as a model example of the use of geospatial databases with environmental indicators. It is an open web application for monitoring the state of forests in near real time, used by researchers around the world. The initiative is implemented by the World Resources Institute (n.d.) in partnership with Google, USAID, the University of Maryland, Esri, Vizzuality, and other scientific, non-governmental, and private organisations. The platform uses satellite data, including from NASA MODIS sensors, as well as image processing algorithms to detect tree cover loss, fires, land use changes and other processes. For experimental confirmation of this study, all six polygons representing the boundaries of the CNNP according to various sources (WDPA, OSM, authors', etc.) were uploaded to GFW. Based on these geospatial layers, analytical data on forest cover, forest loss, forest growth and forest fires were obtained, which are summarised in Table 2, and Figure 6 shows a visualisation of the correlation of the layers according to selected indicators. This made it possible to quantitatively assess how even minor deviations in the polygon area can lead to significant distortions in environmental indicators, which is critically important for reliable environmental monitoring.

Table 2. Boundary consistency and deviations in environmental metrics: Forest loss, fires, and forest gain

Name	Forest loss 2001-2024 (ha)	Deviation in forest loss (%)	Forest loss from fires 2001-2024 (ha)	Deviation in forest loss from fires (%)	Forest gain 2000-2020 (ha)	Deviation in forest gain (%)
Authors' 504	1,430	0.00	11	0	212	0.00
WDPA 612	3,880	171.33	28	154.55	320	50.94
OSM 529	1,600	11.89	16	45.46	222	4.72
OSM 512	1,500	4.90	14	27.27	217	2.36
WDPA 500	1,580	10.49	12	9.09	237	11.79
CNNP 499	1,400	-2.10	11	0	208	-1.89

Source: developed by the authors

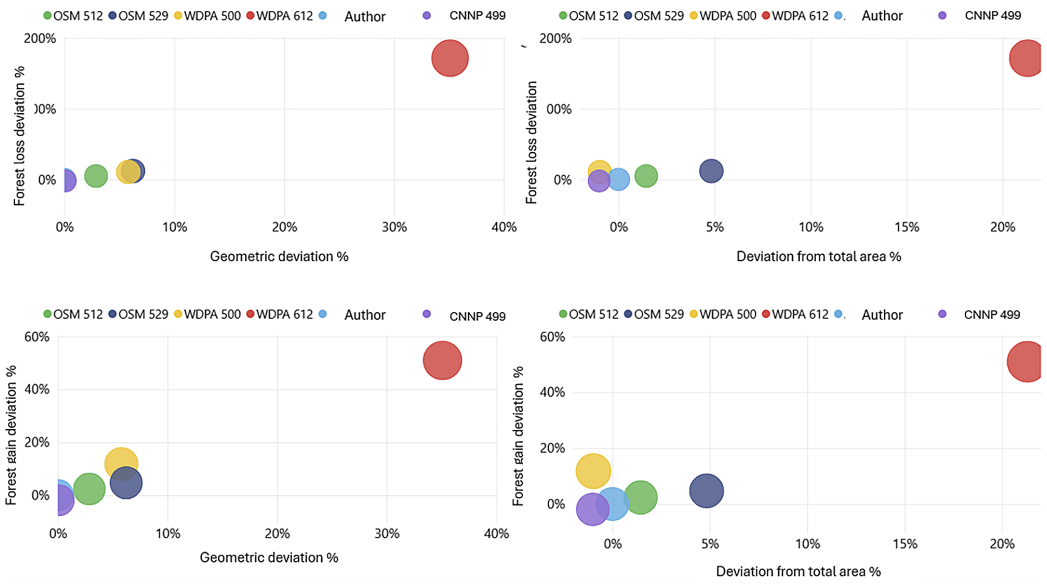

Figure 6. Correlation analysis of the impact of geometric and planar deviations of nature conservation areas on environmental indicators: Forest loss and growth

Source: developed by the authors

To compare environmental indicators for forest loss and gain in the CNNP territory, the WDPA 500 and CNNP 499 geospatial layers were used, with almost identical minimum deviations from the authors' geospatial layer at -0.97% and 1.01%, respectively. However, when the deviation is considered in terms of forest loss, a completely different picture emerges: for WDPA 500, it is 10.49%, and for CNNP 499, it is -2.10%, i.e. a difference in the range of 12.5%; for forest loss from fires, it is 9.09% and 0% respectively, and for forest growth, it is 11.79% and -1.89% respectively. These examples prove that even a small difference in the area and geometry of the polygon (1% in area and 5.8% in geometry for this example) can lead to significant distortions in the calculation of environmental indicators.

Despite minor geometric differences between the OSM 529 and OSM 512 layers compared to the authors' polygon (area deviation is 4.84% and 1.45% respectively, and spatial coincidence is over 98.5%), the analysis of environmental indicators shows significant discrepancies: forest cover losses for OSM 529 exceed the reference value by 11.89%, and for OSM 512 – by 4.90%; forest losses from fires increase by 45.46% and 27.27%, respectively; forest growth also shows deviations – 4.72% for OSM 529 and 2.36% for OSM 512. These results confirm that even minimal discrepancies in area and boundary configuration can lead to significant distortions in the calculation of environmental indicators, especially when assessing forest loss, the impact of fires and forest cover dynamics. Taking the most critical example from WDPA 612, its 21% difference in area and 35% difference in geometry already distorts the indicators for forest loss by 171.33%, forest loss from fires by 154.55% and forest growth by 50.94%. Figure 7 shows an example of a report from Global Forest Watch.

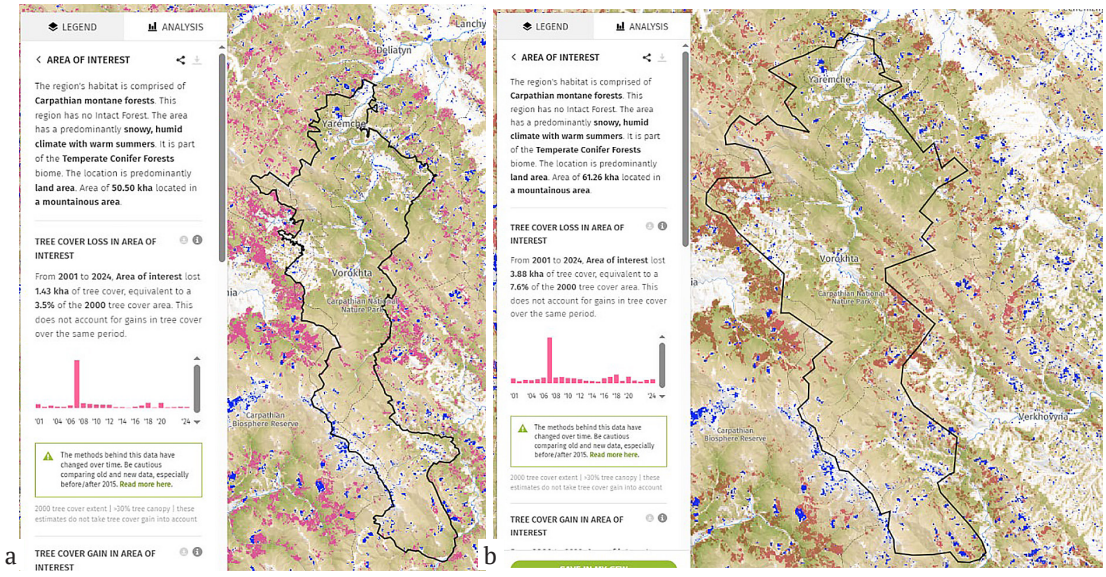

Figure 7. Example of a Global Forest Watch analytical report
Note: a – for the Authors' geospatial layer; b – for the WDPA 612 geospatial layer
Source: developed by the authors based on Global Forest Watch (n.d.)

The results confirm the critical sensitivity of environmental assessments to the accuracy and consistency of the boundaries of nature conservation areas. Even with high spatial overlap (≈98-99%), small differences in area and geometry (1-5%) lead to significant discrepancies in forest loss, growth and fire indicators. This indicates the need to apply unified data collection and processing methods to improve the accuracy of assessments. In addition, such discrepancies can influence management decisions and the development of forest protection strategies. Below is a

comparative analysis with the works of other authors who have researched related topics.

Researchers V. Brusak & V. Shtuglynets (2021) demonstrated that the representativeness of mountain landscape types in the Carpathian NRF largely depends on the quality of the source geodata: the generalisation of contours and the inconsistency of projections lead to systematic biases in the representation of landscape complexes. This is consistent with the findings of this study: polygons with similar areas but different contours (e.g., OSM 512 vs. the authors' layer) show noticeable deviations in ecological metrics (forest loss +4.90%). The difference lies in the focus: V. Brusak & V. Shtuglynets assess landscape representativeness, while this study quantitatively shows how contour errors are transferred to applied ecological indicators through the "Deviation from intersection area (%)" indicator. The study by X. Kang *et al*. (2024) demonstrated that a systematic framework for defining the boundaries of protected areas increases spatial accuracy and territorial integrity, which directly affects the reliability of ecosystem status assessments and the zoning of management priorities. This is consistent with the present approach to metric verification of boundaries and demonstrates the importance of integrated analysis to reduce errors in ecological indicators.

A. Novikov (2021) proposed GIS-oriented zoning (geomorphological and phytogeographical) as a set of operational units consistent with modern digital data. His approach emphasises the need to "align" thematic taxa with reference spatial frameworks. These results confirm this thesis with practical examples: differences in boundary configurations (even for almost identical areas) significantly affect aggregated ecological assessments (e.g., WDPA 500 vs. CNNP 499: deviation in forest loss 10.49% vs. -2.10%). The difference in approaches is that this study focuses on metric verification of boundary consistency between sources before linking them to taxa and indicators. L.V. Shtohryn & D.V. Kasiynchuk (2024) showed the dominance of orographic and climatic factors in the formation of landslide processes, which requires high spatial accuracy of base layers for territorial hazard assessments. The results of this study are consistent: for mountain parks, where boundaries often follow natural boundaries (watersheds, ridges), each "shift" of the boundary line can change both the hazard area and its statistical generalisation. The article adds quantitative granularity to this sensitivity through coincidence metrics and "Deviation from intersection area (%)", demonstrating that geometric boundary errors translate into errors in the assessment of environmental risks and processes. Research by H. Gao *et al*. (2022) shows that spatial modelling of conservation networks and boundary accuracy directly affect the effectiveness of management and prioritisation of areas. This confirms the importance of polygon alignment and metric control for valid spatial conclusions.

Researchers R.L. Kravchynskyi *et al*. (2025) emphasise the standardisation of field methods and systematic monitoring of abiotic components of CNNP, which requires a validated spatial framework. This study provides such a framework: the authors' polygon (50,495 ha) is reduced to the official area, taking into account omitted functionally important areas (the Hoverla Research and Conservation Scientific Department arboretum, the Prut riparian zone, and the peaks of the Chornohora Range). The similarity lies in the requirement for geometry validation. J. Randall *et al*. (2022) demonstrated the effectiveness of integrating field surveys, web GIS, and visualisation tools for invasive species management. The conclusions of this study complement their results: the correctness of boundaries is a prerequisite for valid observation routes, prioritisation of areas and interpretation of spatio-temporal patterns. The examples given in the article (OSM discrepancies 529 due to the inclusion of part of the CBR and the corresponding overestimation of forest loss and fires) illustrate that even a "high-quality" workflow becomes unreliable without verified contours.

The World Database on Protected Areas (n.d.) explicitly points to limitations in data interpretation, particularly cases where objects are represented by points and heterogeneity in the origin of geometries. This is fully consistent with the identification of critical discrepancies: WDPA 612 shows an area deviation of 35% and an overestimation of environmental indicators (forest loss +171.33%; fires +154.55%). Studies by P. Potapov *et al*. (2022) and A. Tyukavina *et al*. (2022) demonstrated a methodology for global detection of tree cover loss based on satellite data and

post-processing algorithms. The results of this study are conceptually consistent: aggregated estimates on the GFW platform are sensitive to the choice of input polygon. This study demonstrates that contours with almost equal areas but different geometric consistency (e.g., WDPA 500 and CNNP 499) yield different loss results (+10.49% vs. -2.10%) and gains (+11.79% vs. -1.89%). This is a practical difference: while P. Potapov *et al*. and A. Tyukavina *et al*. focus on change detection algorithms, this study shows how the geometry of administrative nature conservation boundaries scales the error when aggregating these changes.

In their study, L. Kleinewillinghöfer *et al*. (2022) proposed an unbiased assessment of areas using Copernicus High Resolution Layers and reference data; the approach is consistent with the principle of "agreed area → valid indicators" and can be used as a baseline area validation for the final CNNP boundary model before aggregating losses/gains. Researchers M. Jurišić *et al*. (2023) and O. Chaskovskyy *et al*. (2025) showed that ecological sensitivity zoning schemes and visitor forecasts in national parks depend on the accuracy of the spatial framework; it has been established that small contour discrepancies between sources (OSM vs. authors' layer) can shift management priorities (observation routes, load zoning). Researchers M.S. Parvez & X. Feng (2024) proposed a general method of spatial delimitation combining optimisation and user control; This approach supports the logic of a "quality gate": before linking taxa/boundary indicators, they should be algorithmically reconciled between sources and undergo a metric audit (coincidence, "Deviation from intersection area (%)"). M. Deputat *et al*. (2025) demonstrated, using the Carpathian region as an example, that the interpretation of climate-sensitive indicators (NDSI, snow cover) in the mountains depends on correct zonal binding; the results obtained complement this conclusion with practice: different contours scale the total estimates of changes even for almost equal areas.

Scientists V. Ruschak & I. Chepurnyi (2025) and M. Karabiniuk *et al*. (2025) emphasise that to ensure the accuracy of remote assessments, it is necessary to have a coordinated polygon, since proper coordination of boundaries is a key condition for the stability of results. This confirms the importance of accurately defining the boundaries of protected areas, such as the Carpathian National Nature Park, to ensure the reliability of environmental data and effective monitoring of the state of the environment. The study by X. He & H. Wei (2023) highlights the importance of accurately defining the boundaries of protected areas for biodiversity conservation and effective natural resource management, which is relevant to this study. The authors point to the need to integrate geospatial data through GIS and satellite imagery to improve monitoring accuracy, identify errors in publicly available datasets, and refine park boundaries, which is critical for assessing forest area and land use changes. The results of the study confirm the effectiveness of combining open data, field research, and modern GIS technologies to improve the management of protected areas, which is important for sustainable nature management.

A body of previous research confirms the basic requirement: validated and agreed boundaries are a prerequisite for correct spatial conclusions in mountain parks. The contribution of this study is to operationalise this requirement through the metric "Deviation from intersection area (%)", a systematic audit of multi-source polygons (WDPA, OSM, local CNNP data) and a demonstration of practical implications for monitoring forest loss, fires and growth. The differences between sources (especially WDPA 612) are not "cosmetic": they cause significant distortions in environmental indicators and can lead to misguided management decisions. The proposed approach provides a reproducible, quantitatively sound route to a single verified model of CNNP boundaries and can be replicated for other mountain NRF in Ukraine.

CONCLUSIONS

Accurate delineation of protected areas is critical to ensuring reliable environmental monitoring, especially in complex mountainous terrain such as that found in the Carpathian National Nature Park. Microclimatic zoning, high biodiversity with endemic and rare species, and intense anthropogenic pressure – in particular recreational use and forestry – necessitate maximum accuracy of geospatial data. The use of inaccurate polygons from global databases such as WDPA can lead to erroneous scientific conclusions, incorrect monitoring planning, and errors in international reporting. Even minor deviations in area (1-5%) can

cause significant errors in calculations of indicators such as forest cover loss, forest fire area and forest growth, which in turn affects the quality of environmental assessments and the effectiveness of nature conservation planning.

The authors' research demonstrates the need for local verification of the boundaries of nature conservation areas and proposes an updated CNNP polygon as a reliable tool for further environmental assessments. The authors' polygon (504.95 km^2) with a full intersection (100%) was taken as a reference. The CNNP polygon 499 showed a minimal deviation in area (0.08%) and, accordingly, insignificant deviations in environmental indicators (forest loss -2.10%; growth -1.89%), which confirms the correctness of the locally verified boundaries. On the other hand, geometrically divergent polygons lead to significant shifts in metrics. For OSM 512, with an area difference of 2.88% from the intersection, an increase in forest loss of +4.90% and fire loss of +27.27% is recorded. For OSM 529, with an area difference of 6.24%, forest loss is +11.89% and fire loss is +45.46%. WDPA 612 is particularly indicative: an area difference of 35.10% and overestimated indicators (forest loss +171.33%; fires +154.55%; increase +50.94%), which makes the use of such a contour unacceptable even for overview tasks. At the same time, WDPA 500 (area difference 5.80%) shows moderate but significant deviations (forest loss +10.49%; growth +11.79%), which can change management priorities and interpretations. There was a justified need for local verification of boundaries and the use of the updated CNNP polygon as a reference framework for environmental assessments, spatial planning and reporting. It is recommended to use Sentinel 2 (Copernicus) satellite products for land cover and NDVI change analysis, MODIS and Landsat for long-term series, Global Forest Watch for forest loss assessment, and OpenStreetMap together with local geodata for infrastructure mapping. In total, even 1-5% geometric error can scale indicator errors to >10%, confirming that validated and agreed boundaries are a prerequisite for correct conclusions and effective nature conservation management. Prospects for further research lie in the development of integrated methods for continuous local verification of CNNP boundaries using multi-temporal satellite data, automated GIS algorithms, and field observations to improve the accuracy of environmental monitoring and the soundness of management decisions.

ACKNOWLEDGEMENTS

None.

FUNDING

None.

CONFLICT OF INTEREST

None.

REFERENCES

[1] Brusak, V., & Shtuglynets, V. (2021). Erosion processes of mountain tourist trails in the Chornohora massif (Ukrainian Carpathians). In *International conference of young professionals "GeoTerrace-2021"* (pp. 1-5). Lviv: European Association of Geoscientists & Engineers. doi: 10.3997/2214-4609.20215K3025.

[2] Carpathian Biosphere Reserve. (n.d.). Retrieved from https://kbz.in.ua/en/.

[3] Carpathian National Nature Park. (2024). *Chronicle of Nature, Book XXXIX*. Carpathian National Nature Park.

[4] Carpathian National Nature Park. (n.d.). Retrieved from https://karpatskyi-park.in.ua/en/.

[5] Chaskovskyy, O., Havryliuk, S., Sinkevych, O., Nechepurenko, A., Pelyukh, O., & Rozumovskyi, M. (2025). Leveraging satellite data to support decisionmaking on forest disturbances in "Skole Beskids" National Nature Park. In *Computational methods in systems engineering 2025: Proceedings of the international workshop on computational methods in systems engineering (CMSE 2025)*. Kyiv.

[6] Deputat, M., Terletska, K., Zhupnyk, V., Horishevskyi, P., & Kasiyanchuk, D. (2025). Study of the impact of climate change on tourism activities using remote sensing in the Carpathian region. *Geographia Technica*, 20(2), 15-30. doi: 10.21163/GT_2025.202.02.

[7] European Space Agency. (n.d.). Retrieved from https://surl.li/snorxf.

[8] Gao, H., Xu, J., Zhang, Y., & Liu, X. (2022). An innovative framework on spatial boundary delineation of nature reserves. *Sustainability*, 14(2), article number 12587. doi: 10.3390/su14020587.
[9] Global Forest Watch. (n.d.). Retrieved from https://www.globalforestwatch.org.
[10] He, X., & Wei, H. (2023). Biodiversity conservation and ecological value of protected areas: A review of current situation and future prospects. *Frontiers in Ecology and Evolution*, 11, article number 1261265. doi: 10.3389/fevo.2023.1261265.
[11] Jurišić, M., Plaščak, I., Rendulić, Ž., & Radočaj, D. (2023). GIS-based visitor count prediction and environmental susceptibility zoning in protected areas: A case study in Plitvice Lakes National Park, Croatia. *Sustainability*, 15(2), article number 1625. doi: 10.3390/su15021625.
[12] Kang, X., Du, M., Zhao, L., Liu, Q., Liao, Z., Su, H., Xiang, T., Gou, C., & Liu, N. (2024). Integrity-centered framework for determining protected areas boundary: An application in the China's national park. *Ecological Informatics*, 84, article number 102885. doi: 10.1016/j.ecoinf.2024.102885.
[13] Karabiniuk, M., Saliuk, M., Burianyk, O., Hostiuk, Z., & Lutso, V. (2025). Mapping degradation hotspots of highmountain geocomplexes in Chornohora under recreational pressure around Nesamovyte Lake (Ukrainian Carpathians). In *18th international conference monitoring of geological processes and ecological condition of the environment* (pp. 1-5). Kyiv: European Association of Geoscientists & Engineers.
[14] Kleinewillinghöfer, L., Olofsson, P., Pebesma, E., Meyer, H., Buck, O., Haub, C., & Eiselt, B. (2022). Unbiased area estimation using Copernicus High Resolution Layers and reference data. *Remote Sensing*, 14(19), article number 4903. doi: 10.3390/rs14194903.
[15] Kravchynskyi, R.L., Korchemliuk, M.V., Khilchevskyi, V.K., Tymchuk, J.J., & Stefurak, O.M. (2025). *Study of the natural conditions of the Carpathian National Nature Park: the abiotic aspect*. Ivano-Frankivsk: Foliant.
[16] Nature Conservation Bureau. (n.d.). Retrieved from http://cbr.nature.org.ua/jpg/zapmap.jpg.
[17] Novikov, A. (2021). Developing the GIS-based maps of the geomorphological and phytogeographical division of the Ukrainian Carpathians for routine use in biogeography. *Biogeographia – The Journal of Integrative Biogeography*, 36, article number a009. doi: 10.21426/B636052326.
[18] OpenStreetMap. (n.d.). Retrieved from https://www.openstreetmap.org/#map=5/48.54/31.17.
[19] Parvez, M.S., & Feng, X. (2024). Generic method for social – environmental system boundary delineation – an amalgamation of spatial data integration, optimization, and user control for resource management. *ISPRS International Journal of GeoInformation*, 13(12), article number 447. doi: 10.3390/ijgi13120447.
[20] Potapov, P., *et al*. (2022). The Global 2000-2020 land cover and land use change dataset derived from the Landsat archive: First results. *Frontiers in Remote Sensing*, 3, article number 856903. doi: 10.3389/frsen.2022.856903.
[21] Randall, J., Inglis, N.C., Smart, L., & Vukomanovic, J. (2022). From meadow to map: Integrating field surveys and interactive visualizations for invasive species management in a national park. *ISPRS International Journal of GeoInformation*, 11(10), article number 525. doi: 10.3390/ijgi11100525.
[22] Ruschak, V., & Chepurnyi, I. (2025). Assessment of forest cover dynamics in landslideprone areas of the Ukrainian Carpathians using remote sensing data. In *18th international conference monitoring of geological processes and ecological condition of the environment* (pp. 1-5). Kyiv: European Association of Geoscientists & Engineers. doi: 10.3997/2214-4609.2025510172.
[23] Shtohryn, L.V., & Kasiynchuk, D.V. (2024). Analysis of natural and manmade factors of landslide development in the Carpathian region using GIS. *Scientific Bulletin of the National Mining University*, 5, 93-98. doi: 10.33271/nvngu/2024-5/093.
[24] Tezel, D., Buyukdemircioglu, M., & Kocaman, S. (2021). Accurate assessment of protected area boundaries for land use planning using 3D GIS. *Geocarto International*, 36(1), 96-109. doi: 10.1080/10106049.2019.1590466.
[25] The World Database on Protected Areas. (n.d.) Retrieved from https://surl.lt/gigcwb.
[26] Tyukavina, A., *et al*. (2022). Global trends of forest loss due to fire, 2001-2019. *Frontiers in Remote Sensing*, 3, article number 825190. doi: 10.3389/frsen.2022.825190.

[27] Urbano, F., Viterbi, R., Pedrotti, L., Vettorazzo, E., Movalli, C., & Corlatti, L. (2024). Enhancing biodiversity conservation and monitoring in protected areas through efficient data management. *Environmental Monitoring and Assessment*, 196, article number 12. doi: 10.1007/s10661-023-11851-0.
[28] World Resources Institute. (n.d.). Retrieved from https://surl.li/cijipc.

ГІС-аналіз меж Карпатського НПП: порівняння глобальних і локальних джерел геоданих і важливість для екологічного моніторингу

Петро Насадюк

Аспірант
Львівський національний університет імені Івана Франка
79000, вул. Університетська, 1, м. Львів, Україна
https://orcid.org/0009-0008-8189-4945

Звенислава Мамчур

Кандидат біологічних наук, доцент
Львівський національний університет імені Івана Франка
79000, вул. Університетська, 1, м. Львів, Україна
https://orcid.org/0000-0003-0527-5639

Оксана Тимчук

Науковець
Карпатський національний природний парк
78500, вул. Василя Стуса, 6, м. Яремче, Україна
https://orcid.org/0009-0000-4728-9401

Анотація. Актуальність дослідження полягає у необхідності забезпечення високої точності визначення меж Карпатського національного природного парку (КНПП), що є важливим для достовірності екологічного моніторингу із застосуванням геоінформаційних систем (ГІС) та польових досліджень. Збереження природних екосистем і ефективне управління територією Карпатського національного природного парку неможливі без інтеграції та аналізу просторових даних у геоінформаційних системах, що забезпечують обґрунтоване планування природоохоронних заходів і контроль стану довкілля. Метою статті був аналіз геопросторових наборів даних (полігонів меж) КНПП, доступних у відкритих джерелах та офіційних матеріалах, порівняння їхньої точності та придатності для аналізу екологічних показників, проведення точкової польової верифікації та розробка авторського варіанта уточнених меж парку. У дослідженні проаналізовано можливості використання відкритих геопросторових даних, зокрема супутникових знімків, цифрових моделей рельєфу, а також тематичних шарів платформи Global Forest Watch у середовищах QGIS та ArcGIS. Порівняння різних джерел показало наявність значних помилок у публічно доступних наборах даних, що призводить до викривлень у статистичних оцінках площі лісів, змін землекористування та визначення зон антропогенного впливу. На основі геопросторового аналізу, польових обстежень і співставлення офіційних матеріалів запропоновано авторський варіант уточнених меж КНПП. У роботі наведено приклад того, як неточності меж у глобальних системах моніторингу, зокрема на платформі Global Forest Watch, можуть суттєво змінювати оцінку площ вирубок або деградації лісів. Це підкреслює необхідність постійної перевірки та оновлення геопросторових даних для природоохоронних територій. Результати дослідження доводять, що поєднання відкритих даних, польових досліджень і сучасних ГІС-технологій є ефективним інструментом для підвищення точності аналізу екологічних даних. Запропонований підхід може бути використаний як модель для вдосконалення просторових меж інших природоохоронних територій України, сприяючи сталому природокористуванню та прозорому управлінню екосистемами

Ключові слова: відкриті дані; геопросторовий шар; супутникові знімки; QGIS; ArcGIS

Received: 19.06.2025 Revised: 28.10.2025 Accepted: 27.11.2025 Published: 26.12.2025

BIOLOGICAL SYSTEMS: Theory and Innovation

ISSN 2706-8382
e-ISSN 2706-8390

Journal homepage: https://bioscience.com.ua/en
Vol. 16, No. 4, 2025, 23-39

UDC 639.512:628.16

DOI: 10.31548/biologiya/4.2025.23

Environmental efficiency of recirculating water supply installations in the artificial breeding of noble crayfish

Artem Kravchenko*
Graduate Student
Dnipro State Technical University
51918, 2 Dniprobudivska Str., Kamianske, Ukraine
https://orcid.org/0009-0004-7848-6239

Kateryna Bozhko
PhD in Biological Sciences, Associate Professor
Dnipro State Technical University
51918, 2 Dniprobudivska Str., Kamianske, Ukraine
https://orcid.org/0000-0002-1481-7164

Abstract. The aim of the study was to assess the effectiveness of a closed water-cycle technology that reduces the impact on natural water bodies and promotes the rational use of water resources. Experimental, analytical and statistical methods were used in the study: biometric measurements, hydrochemical monitoring, behavioural observations and correlation analysis. At the beginning of the experiment, the mean length of the juveniles was 1.2 cm and the weight 1.8 g, whereas by the end of the observations these indicators had increased to 5.1 cm and 11.7 g respectively. Survival remained within 85-100%, which indicated optimal cultivation conditions. The water temperature was stably maintained at 21-22°C, and the oxygen concentration fluctuated within 6.8-7.1 mg/l, ensuring normal metabolism of hydrobionts. Hydrochemical analysis showed the effective functioning of the biofilter: the concentration of ammonium nitrogen decreased from 0.35 to 0.07 mg/l, nitrites from 0.15 to 0.03 mg/l, and nitrates from 4.8 to 2.8 mg/l. Water transparency increased from 42 to 55 cm, and pH stabilised at 7.5, which indicated completion of the stage of biological stabilisation of the environment. Behavioural indicators also confirmed successful adaptation: activity of the juveniles increased from 2.1 to 4.6 points, the stress level decreased from 3.8 to 1.8 points, and cannibalism fell to 0.9%. After transfer to a natural water body, crayfish survival was 72%, the mean weight 133 g, and the proportion of females with eggs reached 65%, which confirmed the natural reproductive capacity. Economic calculations showed production profitability of 55.2% at a cost price of 138 UAH/kg and a net profit of 2,050 UAH/m^2/year. Energy consumption of the system was low – 3.6 kWh/day, saving of water resources – 92%, and the environmental effect – a 40% reduction in the catch of natural populations. The practical significance of this study lies in the possibility of using its results for the introduction of energy-efficient technologies for rearing noble crayfish on farms and in industrial enterprises in Ukraine

Keywords: natural populations; survival; hydrobionts; profitability; adaptation

Suggested Citation:
Kravchenko, A., & Bozhko, K. (2025). Environmental efficiency of recirculating water supply installations in the artificial breeding of noble crayfish. *Biological Systems: Theory and Innovation*, 16(4), 23-39. doi: 10.31548/biologiya/4.2025.23.

*Corresponding author

INTRODUCTION

The relevance of this study is determined by the need to conserve and restore natural populations of noble crayfish (*Astacus astacus* L.), which are important components of freshwater ecosystems. These populations perform the function of natural sanitarians of water bodies, taking part in self-purification and regulation of the biological balance. The presence is an indicator of environmental stability, since crayfish are sensitive to pollution and changes in environmental quality. In the last few years (2020-2024), a significant decline in crayfish numbers has been observed, associated with military actions, degradation of water bodies and poaching. In such a situation, the introduction of environmentally safe technologies for rearing hydrobionts, in particular the use of recirculating water supply installations, becomes especially important. This technology minimises water abstraction and the discharge of polluted effluents, ensuring sustainable development of aquaculture. Thus, the development of artificial crayfish rearing in recirculating water supply systems is a promising direction that combines environmental feasibility with economic benefits. The issues addressed in this study are driven by a sharp decline in the numbers of *Astacus astacus* in Ukrainian water bodies, in particular in the Dnipro region, under the influence of anthropogenic and climatic factors. Juveniles do not reach maturity due to water pollution, lack of shelters and increased harvesting. The absence of effective control over fishing increases the risk of local population extinction. In this context, there is a need to create scientifically grounded technologies for laboratory reproduction in recirculating water supply systems capable of ensuring stabilisation of the species, reduction of anthropogenic pressure and development of profitable aquaculture production.

Given the international context of research on aquatic ecosystems, H. Hapich *et al.* (2024) focused on the consequences of the degradation of Ukraine's water resources as a result of military actions. It was emphasised that the restoration of aquatic biocenoses became possible only through the combination of technological and nature-conservation approaches, among which aquaculture was identified as a key compensatory mechanism. Within the framework of sectoral analysis, O. Ishchuk *et al.* (2024) identified trends in the development of crustacean aquaculture in Ukraine and its strategic role in ensuring food security. It was established that the main constraints remained the lack of recirculating water supply installations and of trained specialists for the operation.

Relying on biotechnological data, N. Hrynevych *et al.* (2022) described the potential of culturing *Cherax quadricarinatus* as a way of increasing the economic efficiency of aquaculture. It was shown that introduced species had high growth rates, but the adaptation to local conditions required additional ecotoxicological studies. In the context of experimental work, R.E. Duffy *et al.* (2025) presented the results of successful incubation and rearing of *Cherax tenuimanus* in closed-type systems. It was established that controlled hydrochemistry of the environment ensured survival above 90%, confirming the effectiveness of recirculating water supply technologies. On the basis of field observations, O.M. Edwards *et al.* (2025) analysed the impact of hydrological and climatic factors on the dynamics of aquatic organism populations. It was determined that fluctuations in temperature and oxygen levels significantly affected behaviour and reproductive activity. From the standpoint of ecological monitoring, S. Hudina *et al.* (2022) developed a conceptual model for regulating the numbers of signal crayfish (*Pacifastacus leniusculus*) as an invasive species. It was established that effective management of invasive populations requires the combination of environmental monitoring, biocontrol and maintenance of the stability of autochthonous species, including *Astacus astacus*.

Taking into account modern trends in tropical aquaculture, S.S. Lee *et al.* (2025) described the rearing of *Phallusia nigra* in open and closed water supply systems. It was established that closed systems provide stable environmental parameters and increase the viability of hydrobionts. On the basis of experimental data, L. Qin *et al.* (2023) showed that shelters in the rearing environment of *Procambarus clarkii* reduced stress and cannibalism at high stocking density. A positive effect of such elements on larval survival and production profitability was revealed. In terms of international aquaculture experience, M.O. Agbugui *et al.* (2025) summarised fish and crustacean farming practices in Nigeria. The importance of combining

biotechnological methods and socially oriented management models was emphasised. From the standpoint of genetic control, F. Oficialdegui *et al.* (2025) studied variability of *Procambarus clarkii* populations in natural and ornamental systems. Risks of introgression were identified, requiring enhanced control over the import of invasive species.

Within a socio-ecological approach, R. Mohammed *et al.* (2021) described the Freshwater Lobster Production – Green Aquaponics Perennial System for rearing freshwater lobsters in low-income communities. The effectiveness of biofiltration and the stability of local production were demonstrated. On the basis of technical developments in European aquaculture, J. Hinchcliffe *et al.* (2022) summarised the experience of rearing *Homarus gammarus* and identified key technical requirements for automated systems. It was noted that modernisation of equipment is critical for the development of crayfish farming. Issues of long-term adaptation of crayfish reared in recirculating water supply systems after transfer to natural water bodies, and the impact of biofiltration parameters on the formation of stable reproductive groups in artificial populations, remain insufficiently studied. The aim of the study was to assess the effectiveness of recirculating water supply technology for rearing noble crayfish (*Astacus astacus* L.) under conditions close to natural ones, as an environmentally safe method that reduces the impact on natural water bodies and ensures careful use of water resources. The objectives of the study were to determine the dynamics of growth and survival of crayfish juveniles in a recirculating water supply system, to analyse changes in hydrochemical indicators and behavioural reactions of individuals, and to assess the adaptive potential and economic efficiency of rearing under controlled conditions.

MATERIALS AND METHODS

The study was conducted in 2024 (March-August) in the Dnipro district at an experimental hydrobiological laboratory equipped with a recirculating water supply installation for rearing noble crayfish (*Astacus astacus* L.). This species is autochthonous to the Dnipro basin and well adapted to temperate continental conditions, which determined its selection as the object of the study. It is characterised by high viability in recirculating water supply systems, considerable nutritional value and commercial appeal, which makes it optimal for experiments on population restoration and economic evaluation of aquaculture technologies. The experiment was carried out under the supervision of a PhD in Biological Sciences with more than 10 years' experience in aquaculture. Three specialists took part in the observations – two biologists with higher education and one hydrobiological technician, all of whom had undergone prior training in measurement methodology and statistical data recording. The keeping of animals and all experimental manipulations were carried out in accordance with the provisions of the Law of Ukraine No. 249 (2012). In addition, the requirements of the European Commission (2021) on ethics and data protection and of DSTU EN ISO/IEC 17025:2019 (2019) were observed.

The climate of the area is characterised by temperate continental conditions, with a typical mean daytime temperature of +25-28°C in summer. For the experiment, a recirculating water supply installation was used, consisting of three polymer tanks with a volume of 250 l each, a common biofiltration unit with expanded clay filler, a circulation pump Eheim CompactON 1000 (Germany) and a compressor Hailea ACO-318 (China). The water temperature was maintained at 21-2 °C with a Tetra HT 200 thermostat (Germany), and lighting was regulated in a 14:10 (light/dark) regime using Osram LED lamps (Germany).

Separate control tanks were not used, since all three vessels were combined in a single recirculation circuit and functioned as a single technological system. This ensured identical hydrochemical conditions in all sections and made it impossible to create an independent control within one module. Hydrochemical parameters (pH, oxygen, ammonium, nitrites, nitrates) were analysed as integral indicators of the whole system, while biometric and behavioural data were recorded separately for each tank and then averaged, which made it possible to minimise random variations and obtain a representative assessment of the influence of conditions on the growth and behaviour of noble crayfish *Astacus astacus* L.

The observation period lasted six months, with monthly measurements carried out by two hydrobiologists. At the start of the experiment, juveniles of *Astacus astacus* aged 1-2 months,

obtained after laboratory selection of postlarvae, were used. Initial biometric parameters were 1.2 ± 0.1 cm and 1.8 ± 0.3 g, which corresponded to the early juvenile stage of development. The total number of individuals in the experiment was 180 specimens, evenly distributed among the three tanks (60 individuals in each). This ensured equal stocking density and allowed the dynamics of the population to be monitored in terms of the general recirculation system.

For monthly monitoring, 60 individuals were selected each time, 20 from each tank, which made it possible to carry out a weighted analysis without disrupting the general population structure. Mean body length (cm) was measured with a Mitutoyo 500-196-30 digital calliper (Japan) with an accuracy of ±0.01 cm, and mean weight (g) with Radwag WTB 200 laboratory scales (Poland) with an accuracy of ±0.01 g. Water temperature (°C) was recorded with a Testo 108 thermometer (Germany), and the level of dissolved oxygen (mg/l) with a WTW Oxi 3310 electrochemical oximeter (Germany). Survival (%) was calculated according to formula (1):

$$S = \frac{N_2}{N_1} \times 100, \quad (1)$$

where N_1 – the number of individuals at the beginning of the period and the N_2 – number of individuals after completion of the observation period. Measurements were taken simultaneously by two researchers, and a third recorded the indicators in a digital observation log. The data obtained were checked by cross-control.

Water quality control was carried out twice a month. Measurements were taken by a hydrobiological technician under the supervision of the senior researcher. The concentration of ammonium nitrogen (NH_4^+, mg/l) was determined by the phenol-hypochlorite method according to APHA (2017) using a Hach DR/890 photocolorimeter (USA). Nitrites (NO_2^-, mg/l) were determined by the Griess reaction (λ = 540 nm), and nitrates (NO_3^-, mg/l) by the cadmium reduction method on the same device. The pH value was measured with a Hanna Instruments HI98129 portable pH-meter (USA), and transparency (cm) by a Secchi disc 20 cm in diameter with a white scale. Data were averaged over three parallel samples taken by one observer, while another carried out the analysis and verification of the results.

Behavioural observations were carried out daily for 20 minutes twice a day – in the light (10:00) and dark (22:00) periods, corresponding to the natural daily rhythms of *Astacus astacus*. Three specialists took part in the observation group: the chief biologist, an assistant responsible for video recording and a technician responsible for registration of parameters. Video recording was carried out with a Canon EOS 250D camera (Japan), and further analysis of behavioural characteristics was performed using Tracker Video Analysis v6.1 software (USA). Behavioural observations were carried out in parallel with standardised feeding, since feeding activity is a key behavioural indicator. Feeding was carried out twice a day – at 09:00 and 21:00. The main diet consisted of sinking high-protein compound feed (38-42% protein) in 2-3 mm pellets, which ensured even delivery of nutrients. Additionally, every other day natural animal feeds (minced shrimps or fish) were added, which increased feeding motivation and stimulated growth. The mean daily ration was 2.5-3% of total biomass; feed was applied locally in the centre of the tank to control even consumption. After 10-12 minutes, the remains were collected and weighed, which made it possible to assess the degree of feed utilisation and avoid its accumulation in the system.

Activity in the light period was assessed on a five-point scale (1 – low, 5 – high); two independent observers carried out parallel assessments with subsequent verification of discrepancies. Cannibalism (%) was determined visually by the proportion of individuals with injuries after each month of the experiment. Reaction to feed was recorded with a Casio HS-3V-1R stopwatch (Japan), measuring the time from feed application to the approach of the first individual. Aggressiveness was assessed by counting the number of contacts (claw strikes, chases) per hour under reproducible conditions. Stress level was determined on a five-point scale (1 – complete calm, 5 – panic activity). Assessment was carried out simultaneously by two experts, and discrepancies of more than 0.5 points were reconciled after reviewing the video archive.

After completion of the laboratory stage of the study, during which 180 juveniles (60 per tank) were kept in the three tanks, the experiment was continued under natural conditions to assess the adaptive capacity of crayfish to a semi-natural

environment. For the field stage, 60 individuals were selected and transferred to a controlled section of a natural pond with an area of 50 m^2 and an average depth of 1.2 m. This ensured the representativeness of the sample and made it possible to observe the behaviour and physiological changes of individuals reared in the recirculating water supply installation.

The duration of field observations was six months, during which two biologists carried out monthly control catches. Survival was determined according to formula (1), and mean weight was measured with Radwag WTB 200 laboratory scales, which ensured comparability of data with the laboratory stage. Activity was assessed on a five-point scale, cannibalism by the proportion of injured individuals, and stress level by the duration of immobility after mechanical stimulation, recorded with a Casio HS-3V-1R stopwatch. The proportion of females with eggs was determined by the presence of eggs on the pleopods during catches. The content of dissolved oxygen (mg/l) in the bottom layer was measured with a WTW Oxi 3310 oximeter. Behavioural reactions to natural stimuli such as changes in light, current intensity and structure of natural shelters were additionally recorded.

Economic indicators were determined on the basis of actual experimental cost data and market prices obtained from open sources – Agro-Ukraine (n.d.). The duration of the rearing cycle (months) was determined according to the experimental schedule; survival to the end of the cycle (%) – using formula (1), as in the previous stages. The mean weight of marketable crayfish (g) was established on the basis of the control catch of the last month. Among the indicators, the environmental efficiency of the system was also assessed, in particular saving of water resources (%) and reduction of catch of natural populations (%), which was determined by comparison with traditional pond technologies.

Stocking density was maintained at 12 individuals per 1 m^2. To determine this indicator, a measuring frame with an area of 1 m^2, model ARF-1000 (Korea Measuring Instruments, Republic of Korea), was used; it was installed on the bottom of the tank and the individuals within its boundaries were counted directly. Accounting was carried out separately in each tank, followed by calculation of the mean value. Feed costs (UAH/kg of gain) were calculated as the ratio of feed consumed to live weight gain. The cost of rearing 1 kg of crayfish (UAH) was calculated by summing the costs of feed, energy, water, preparations, equipment, and labour. The market sale price (UAH/kg) was determined according to the mean wholesale price for live crayfish on the Ukrainian market (Agro-Ukraine, n.d.). The expected net profit (UAH/m^2/year) was calculated according to formula (2):

$$P = (Q \times C_p) - C_s, \quad (2)$$

where Q – the quantity of products obtained (kg/m^2); C_p – the market sale price (UAH/kg); C_s – the total costs (UAH/m^2/year). The profitability level (%) was determined according to formula (3):

$$R = \frac{P}{C_s} \times 100, \quad (3)$$

where P – net profit (UAH/m^2/year) and C_s – the total costs (UAH/m^2/year). Energy consumption of the installation (kWh/day) was determined from the readings of a Legrand CX^3 412507 meter (France). Saving of water resources (%) was calculated by comparing the volume of water used in the recirculating water supply installations with control pond technologies according to formula (4):

$$E = \left(1 - \frac{V_{CWS}}{V_{pond}}\right) \times 100, \quad (4)$$

where V_{CWS} – the volume of water used in the recirculating water supply installation; V_{pond} – the mean volume of water used in traditional pond technology.

The potential for expansion of production (ha) and the expected annual production (t/ha) were calculated according to stocking density standards and mean productivity. The environmental effect (%) was determined as a reduction in the catch of natural populations compared with baseline statistical data of the State Agency for Land Reclamation, Fisheries, and Food Programmes of Ukraine (n.d.). All results were processed by methods of variation statistics using Statistica 12.0 software (USA). To determine the relationships between growth, survival, hydrochemical indicators and behavioural characteristics, correlation analysis with the Pearson coefficient (r) was applied. The results were presented as mean value ± standard error (M ± SE) with a significance level of $p < 0.05$.

RESULTS

Growth pattern and stability of survival under laboratory rearing conditions

Recirculating aquaculture systems in 2020-2024 are regarded as one of the most effective directions of sustainable aquaculture development, based on the principles of environmental safety and control of environmental quality. Within the framework of the study, this technology was used to create an artificially regulated ecosystem in which all physicochemical parameters of the water were maintained at a stable level. This approach provided consistency between the metabolic processes of hydrobionts and the microbiological self-purification cycles, which minimised dependence on natural water bodies and made it possible to avoid fluctuations in hydrochemical indicators under the influence of external factors.

Control of temperature, oxygen concentration, ammonium nitrogen, nitrites, and nitrates provided stable conditions for the growth and development of juvenile noble crayfish. The biofiltration and aeration system maintained water purification in a closed cycle, significantly reducing the need for fresh water intake. The dynamics of growth and survival of individuals over a six-month period of rearing in recirculating aquaculture systems are presented below (Table 1).

Table 1. Dynamics of growth and survival of crayfish juveniles in the recirculating aquaculture system

Observation period	Mean length, cm	Mean weight, g	Water temperature, °C	Oxygen level, mg/l	Survival, %
Initial (1 week)	1.2 ± 0.1	1.8 ± 0.3	21	7.1	100
1 month	2.4 ± 0.2	4.2 ± 0.4	21.5	6.9	96
2 months	3.1 ± 0.3	6.7 ± 0.5	22	6.8	93
3 months	3.8 ± 0.2	8.9 ± 0.6	21.8	7	90
4 months	4.4 ± 0.3	9.8 ± 0.5	21.5	6.8	88
5 months	4.9 ± 0.4	10.6 ± 0.4	21	6.9	86
6 months	5.1 ± 0.3	11.7 ± 0.5	21	6.8	85

Source: developed by the authors on the basis of formula 1

At the initial stage of the experiment (week 1) the mean length of the juveniles was 1.2 cm and the weight 1.8 g, which corresponded to the post-larval development phase. During this period, survival was 100%, with no losses and no deviations in behaviour. The water temperature was maintained at 21°C, and the concentration of dissolved oxygen at 7.1 mg/l, which created optimal conditions for metabolic processes and initial adaptation to the conditions of the recirculating aquaculture systems. Hydrochemical stability was ensured by the continuous operation of the biofiltration unit, which prevented the accumulation of nitrogenous compounds and contributed to the formation of a natural microbial balance in the water. At this stage, the individuals were characterised by moderate activity, even feed intake and the absence of aggressive behaviour. In the middle of the experiment (month 3) a period of intensive growth was observed. The mean body length of individuals reached 3.8 cm and the weight 8.9 g, which indicated efficient feed conversion and optimal use of energy resources. The water temperature remained stable (21.8°C), the oxygen level 7 mg/l, and the survival rate decreased to 90%, which indicated the onset of natural selection among the juveniles. Behavioural observations showed a stable feeding response – the time taken to approach the feed almost halved compared with the first month, and the level of aggression gradually decreased. The juveniles actively formed a chitinous exoskeleton, which increased the need for calcium and a stable pH of the environment. The biofiltration system effectively maintained the nitrite level below 0.05 mg/l, which prevented a toxic effect on the individuals. The uniformity of growth was noted – the distribution by length and weight had minimal variation, which confirmed identical rearing conditions in all tanks. At the final stage of the study (month 6) the mean length of individuals was 5.1 cm, the weight 11.7 g, and survival decreased to 85%, which is a high indicator for closed systems without water replacement. The temperature remained within 21°C and the dissolved oxygen content at 6.8 mg/l, which ensured stable respiration even with increasing biomass. A slowing

of growth rates was observed, associated with the attainment of the maximum stocking density. Behavioural reactions remained calm, with no signs of stress, panic activity or cannibalism. Biological equilibrium was maintained by the formed biofilm, which acted as a natural filter holding nitrifying bacteria. The conditions of the system remained stable throughout the experiment, as confirmed by the absence of pH fluctuations (7.3-7.5) and the preservation of water transparency.

Over the six months, a clear growth pattern was observed: rapid weight gain during the first three months and a gradual slowdown of development rates after the fourth month, which corresponds to the typical biological dynamics of the species *Astacus astacus* L. Survival rates decreased from 100 to 85%, but remained within the norm for closed hydro-systems. The hydrochemical parameters confirmed the stability of the technology – the concentrations of ammonium nitrogen gradually decreased from 0.35 to 0.07 mg/l, nitrates from 4.8 to 2.8 mg/l, and water transparency increased from 42 to 55 cm, which indicated the efficient operation of the biofiltration complex. The results obtained demonstrated the effectiveness of the conditions created in the recirculating aquaculture system for rearing crayfish juveniles. The stability of water parameters, constant aeration and the absence of external influences ensured a high level of viability, and the growth dynamics confirmed the physiological norm of development without signs of stress. Thus, the experiment confirmed the feasibility of using the recirculating aquaculture system as a basis for artificial reproduction and conservation of stable populations of noble crayfish under controlled conditions.

Stability of hydrochemical parameters in the biofiltration system

Hydrochemical monitoring of recirculating aquaculture systems is a key stage in assessing the effectiveness, since water quality determines the viability of hydrobionts, the stability of the microbiocenosis and the level of environmental safety of the installation. Environmental conditions in recirculating aquaculture systems are constantly changing under the influence of biological processes such as metabolism, mineralisation of organic compounds and nitrification. Therefore, determining the dynamics of the main water indicators – ammonium nitrogen, nitrites, nitrates, acidity, and transparency – makes it possible to trace the sequence of the formation of biological equilibrium in the system (Table 2).

Table 2. Dynamics of hydrochemical indicators in the recirculating aquaculture system

Observation period	NH_4^+, mg/l	NO_2^-, mg/l	NO_3^-, mg/l	pH	Transparency, cm
Initial (1 week)	0.35 ± 0.02	0.15 ± 0.01	4.8 ± 0.3	7.1	42
1 month	0.22 ± 0.02	0.09 ± 0.01	3.6 ± 0.2	7.2	46
2 months	0.15 ± 0.01	0.06 ± 0.01	3.2 ± 0.3	7.3	49
3 months	0.11 ± 0.01	0.05 ± 0.01	3.1 ± 0.2	7.4	50
4 months	0.09 ± 0.01	0.04 ± 0.01	3 ± 0.3	7.4	52
5 months	0.08 ± 0.01	0.03 ± 0.01	2.9 ± 0.3	7.5	53
6 months	0.07 ± 0.01	0.03 ± 0.01	2.8 ± 0.2	7.5	55

Source: developed by the authors

Over the six months of the experiment in the recirculating aquaculture system, a clear trend of improvement in the main hydrochemical indicators was observed, which indicated stabilisation of the nitrogen cycle and effective operation of biofiltration. The content of ammonium nitrogen (NH_4^+) decreased from 0.35 mg/l at the beginning of the experiment to 0.07 mg/l at the end, i.e., more than fivefold. Nitrites (NO_2^-) showed a similar dynamics, with the concentration decreasing from 0.15 to 0.03 mg/l, which indicated active transformation of reduced forms of nitrogen and the full functioning of nitrifying microflora. The final products of mineralisation – nitrates (NO_3^-) – decreased from 4.8 to 2.8 mg/l, which reflected the gradual establishment of a closed nitrogen cycle and a reduction in the load on the biofilter. The pH rose from 7.1 to 7.5, which indicated a reduction in the amount of free carbon dioxide and strengthening of the buffering system of the aquatic envi-

ronment. Water transparency increased from 42 to 55 cm (by approximately 30%), which confirmed a decrease in the concentration of suspended organic particles and increased efficiency of self-purification processes.

The overall dynamics of the indicators shows that the main changes occurred during the first three months of the system's operation, when the biofilm was formed and the microbiological community stabilised. In the subsequent period the recirculating aquaculture system operated in a mode of biological equilibrium, providing low concentrations of ammonium, nitrites and nitrates, stable pH and high-water transparency. This confirms that the recirculating aquaculture system effectively maintains water quality without additional replacement, providing ecologically balanced conditions for rearing hydrobionts.

Formation of social structure and behavioural equilibrium of the population

The behavioural characteristics of crayfish juveniles in recirculating aquaculture systems reflect the processes of adaptation, socialisation and stabilisation of the internal structure of the population. The study of these reactions is important for understanding how technological conditions influence the welfare of hydrobionts and the effectiveness of rearing. During the experiment a consistent decrease in aggression and cannibalism, a reduction in reaction time to feed and a decrease in manifestations of stress were traced, which indicated stabilisation of social behaviour. The main attention was paid not to individual numerical fluctuations, but to trends and cause-and-effect relationships that explain the formation of a harmonious behavioural structure of the group (Table 3).

Table 3. Dynamics of behavioural traits of crayfish juveniles in the recirculating aquaculture system

Observation period	Activity in daylight (points, 5-point scale)	Cannibalism, % of individuals	Reaction to feed (approach time, s)	Aggressiveness (number of contacts/hour)	Stress level (visual assessment, points)
1 month	2.1 ± 0.3	4.8	38 ± 6	7.5 ± 1.1	3.8 ± 0.4
2 months	2.7 ± 0.2	3.6	30 ± 5	6.1 ± 0.8	3.3 ± 0.3
3 months	3.4 ± 0.3	2.2	24 ± 4	4.8 ± 0.6	2.8 ± 0.3
4 months	3.9 ± 0.2	1.5	20 ± 3	3.5 ± 0.5	2.3 ± 0.2
5 months	4.3 ± 0.2	1.1	17 ± 3	2.9 ± 0.4	2 ± 0.2
6 months	4.6 ± 0.1	0.9	15 ± 2	2.5 ± 0.3	1.8 ± 0.2

Source: developed by the authors

Comparative analysis of the behavioural indicators of juvenile noble crayfish over a six-month period of rearing in recirculating aquaculture systems revealed clear relationships between the level of activity, aggression, stress, and reaction to feed, which indicate the gradual stabilisation of the ethological structure of the population. Already at the initial stage activity was 2.1 ± 0.3 points, accompanied by high stress (3.8 ± 0.4 points), significant aggressiveness (7.5 ± 1.1 contacts/hour) and a delayed reaction to feed (38 ± 6 s). This combination of indicators reflected the population's inability to exhibit coordinated behaviour: the animals spent energy on searching for shelters and territorial clashes, which intensified cannibalism (4.8%).

In the middle of the observation period these indicators changed synchronously: an increase in activity to 3.4 ± 0.3 points was accompanied by a decrease in stress to 2.8 ± 0.3 points, a reduction in reaction time to 24 ± 4 s and an almost twofold decrease in aggressiveness – to 4.8 ± 0.6 contacts/hour. At the same time, the proportion of cannibalism decreased to 2.2%. These unidirectional dynamics indicated the formation of primary social organisation: stabilisation of territorial boundaries, synchronisation of movements and coordinated response to feeding. The gradual improvement of the oxygen regime and water purification in the recirculating aquaculture systems contributed to the reduction of irritating factors, which was directly reflected in behavioural reactions.

In the final phase, the most pronounced consolidation of parameters was observed. Activity reached 4.6 ± 0.1 points and stress decreased to 1.8 ± 0.2 points, which indicated physiological stability. Reaction time shortened to 15 ± 2 s and aggressiveness to 2.5 ± 0.3 contacts/hour, i.e., three

times less than at the beginning of observations. Cannibalism dropped to 0.9%, which in fact corresponds to isolated cases. The combination of these indicators demonstrated the transition of the population to a mature social structure: movements became orderly, individuals-maintained distance, and behaviour during feeding was collectively coordinated.

The increase in activity (from 2.1 to 4.6 points) was accompanied by a systematic reduction in aggression (from 7.5 to 2.5 contacts/hour), stress (from 3.8 to 1.8 points) and reaction time to feed (from 38 to 15 s). The relationships between the indicators show that stabilisation of the environment in the recirculating aquaculture system created conditions in which the behavioural energy of individuals ceased to be spent on conflicts and territorial defence and was directed towards growth, feeding and social interaction. This confirms that the recirculating aquaculture system provides an ethologically comfortable space in which the population forms a stable and predictable behavioural structure.

Indicators of physiological adaptation after transfer to the natural environment

Adaptation of artificially reared noble crayfish to natural conditions is a key stage in assessing the effectiveness of recirculating aquaculture technologies. The transition from a controlled environment to a natural water body is accompanied by changes in behavioural, physiological and reproductive indicators, which reflect the level of ecological plasticity of the species. Analysis of these processes makes it possible to determine how successfully individuals reproduced in laboratory conditions are able to maintain a stable population structure in the natural environment. An important aspect is observation of survival, growth, behavioural activity, stress level and frequency of cannibalism, since these parameters indicate the state of adaptation mechanisms (Table 4).

Table 4. Dynamics of the main indicators of crayfish adaptation after transfer to a water body

Indicator	1 month	2nd month	3rd month	4th month	5th month	6th month
Survival, %	100	95	90	84	78	72
Mean weight, g	115 ± 4	118 ± 5	122 ± 5	126 ± 6	129 ± 6	133 ± 7
Activity (5-point scale)	3.1 ± 0.2	3.4 ± 0.2	3.8 ± 0.2	4.1 ± 0.2	4.4 ± 0.1	4.6 ± 0.1
Cannibalism, %	0	1.8	2.9	3.1	2.7	2.4
Stress level (points)	2.8 ± 0.3	2.3 ± 0.2	2 ± 0.2	1.9 ± 0.2	1.8 ± 0.1	1.7 ± 0.1
Proportion of females with eggs, %	-	-	15	35	55	65
Dissolved oxygen, mg/l	7.8 ± 0.4	7.6 ± 0.3	7.4 ± 0.3	7.3 ± 0.3	7.2 ± 0.3	7 ± 0.2

Source: developed by the authors on the basis of formula 1

After the transfer of older crayfish, previously reared in the recirculating aquaculture system, to a natural water body, a consistent dynamic of physiological and behavioural adaptation was observed. At the beginning of the observations, survival was 100%, mean weight 115 ± 4 g, activity 3.1 ± 0.2 points and stress level 2.8 ± 0.3 points. A stable oxygen background (7.8 ± 0.4 mg/l) and the presence of natural shelters ensured the absence of cannibalism and created favourable conditions for acclimatisation. High motor coordination during evening and night periods indicated the preservation of the natural ethological rhythm after transfer from the controlled environment.

During the third month survival naturally decreased to 90%, which reflected typical adaptation losses in open water bodies, but at the same time progress in physiological parameters was noted. The mean weight increased to 122 ± 5 g, activity to 3.8 ± 0.2 points and stress level decreased to 2 ± 0.2 points. The oxygen regime remained stable (7.4 ± 0.3 mg/l), which supported normal metabolism. Cannibalism was 2.9%, occurring mainly during moulting periods. At this stage, the first females with eggs appeared (15%), which indicated restoration of reproductive functions and adaptation to the natural cycle.

By the end of the sixth month, the population had acquired features of ecological stability. Survival was 72%, mean weight increased to 133 ± 7 g, activity to 4.6 ± 0.1 points and stress level decreased to 1.7 ± 0.1 points. The

proportion of cannibalism decreased to 2.4%, which corresponds to the natural level for adult populations. The concentration of dissolved oxygen remained at 7 ± 0.2 mg/l. The most important indicator was a significant increase in the proportion of females with eggs to 65%, which confirmed complete reproductive adaptation and maturation of gonads in the natural environment. Behavioural reactions at the end of the period became clearly structured: crayfish maintained territorial distances, avoided conflicts and responded to natural stimuli (current, light, food base) without signs of disorientation. Synchronisation of activity and reduction of stress reactions confirmed the successful formation of a balanced behavioural structure of the population.

Assessment of the economic performance of the intensive rearing technology

The development of crayfish rearing technologies in recirculating aquaculture systems opens up new opportunities for creating efficient, ecologically balanced aquaculture models. Such systems make it possible to combine the stability of production processes with minimal impact on the environment, ensuring a steady increase in biomass under controlled parameters of the aquatic environment. A comprehensive assessment of biological, technological and economic aspects makes it possible to determine the optimal operating conditions of recirculating aquaculture systems, ensuring a combination of high productivity, low energy consumption and environmental sustainability of production (Table 5).

Table 5. Main indicators of the economic and environmental efficiency of the experiment

Indicator	Value	Interpretation/comment
Duration of the rearing cycle, months	12	Full annual cycle with two phases: artificial on-growing and semi-natural holding
Survival to the end of the cycle, %	72	High indicator for closed systems without water replacement
Mean weight of marketable crayfish, g	133 ± 6	Optimal size for sale on the market
Number of individuals per 1 m², pcs	12	Stocking density without signs of stress
Feed costs, UAH/kg of gain	48	Economically acceptable level when using compound feeds
Cost of rearing 1 kg, UAH	138	Includes costs of feed, electricity, water, and equipment depreciation
Mean market sale price, UAH/kg	310	According to prices on the domestic market for live crayfish
Expected net profit, UAH/m²/year	2,050	Calculated taking into account profitability above 55%
Profitability level, %	55.2	High economic efficiency with minimal environmental risks
Energy consumption of the installation, kWh/day	3.6	Low indicator due to water recirculation
Saving of water resources, %	92	Compared with the traditional pond method
Potential for expansion of production, ha	0.5-1	For farm-level production while maintaining profitability
Expected annual production, t/ha	3.2	With a two-cycle turnover of individuals
Environmental effect (reduction of catch from nature), %	40	Contributes to reducing pressure on wild populations and biodiversity of water bodies

Source: developed by the authors based on Agro-Ukraine (n.d.), DSTU EN ISO/IEC 17025:2019 (2019), and formulae 1-4

The total duration of the rearing cycle was 6 months. Such a structure of the cycle made it possible to maintain continuity of the process from the moment of stocking juveniles to obtaining marketable products, ensuring complete closure of the technological chain. The survival rate to the end of the cycle was 72%, which is high for recirculating systems. This indicated the efficient operation of the biofiltration system, optimal water parameters and the absence of toxic load. High survival ensured population stability, reduced costs for restocking juveniles and lowered the cost price of the products. The mean weight of marketable crayfish was 133 ± 6 g, which corresponds to market standards for the sale of live product in restaurants or on wholesale markets. Such individuals have an optimal ratio of meat to shell, which increases the commercial attractiveness.

A stocking density of 12 individuals per 1 m² ensured a balance between biological productivity and environmental comfort. This density made it possible to avoid stressful situations, reduced the risk of cannibalism, and did not require excessive filtration. At higher stocking densities, as field

observations show, the frequency of contacts between individuals increases, which can negatively affect growth and survival. Special attention was paid to feed costs, which were 48 UAH/kg of gain. This indicator is considered economically acceptable for aquaculture, as it provides a feed-to-gain ratio close to 1.5:1. The reduction in costs became possible due to the use of compound feeds with increased energy value and the remnants of natural food in the water body. The cost of rearing 1 kg of crayfish was UAH 138, which included the costs of feed, electricity, maintenance of the filtration system, water, and equipment depreciation. Even under conservative calculations, with a mean market sale price of 310 UAH/kg, profitability remained considerable. Thus, net profit was about 2,050 UAH/m²/year, and profitability reached 55.2%. This level of efficiency makes it possible to classify the technology as highly profitable for small-scale farming. Energy efficiency of the system was of particular importance. The average energy consumption of the installation was only 3.6 kWh/day, which is a low indicator for aquaculture facilities with constant aeration. This became possible due to the design of the biofilter with natural aeration and water recirculation. Thus, operating costs for electricity remained minimal, which additionally increased the overall economic feasibility.

An important indicator was the saving of water resources, which reached 92% compared with traditional pond technologies. This meant that, due to the closed cycle of purification and reuse of water, the system functioned practically without losses. Such an approach made it possible not only to reduce costs, but also to ensure compliance with modern environmental water-saving requirements. From the point of view of scaling up, the potential for expansion of production was 0.5-1 ha for farm-level production while maintaining profitability. Calculations showed that at such a scale it is possible to achieve an annual production of up to 3.2 t/ha, i.e., about 3,200 kg of marketable crayfish with a two-cycle turnover of individuals. This made it possible to transform the experimental model into a stable farm with a fully closed biotechnological process.

Assessment of the environmental effect showed that the introduction of the system can reduce the catch of natural crayfish populations by approximately 40%, which has a direct positive impact on river and lake ecosystems. A reduction in poaching pressure, in turn, contributes to the restoration of natural spawning grounds and improvement of biodiversity. In addition, water leaving the system after biofiltration did not contain excess organic substances, which prevented eutrophication or deterioration of the quality of natural waters. An important indicator of environmental sustainability is the energy intensity of the process, which remained within natural balances and did not require constant use of fuel resources. Owing to this, the system had the potential to be integrated into local energy-saving programmes, including power supply from solar panels or biogas plants. Thus, the comprehensive assessment proved that the proposed technology combined high biological productivity, a minimal level of water consumption, low energy costs and significant profitability. It can be adapted to the conditions of different regions – from private households to industrial farm systems. The overall result showed that a cycle duration of 12 months, survival of 72%, profitability of 55%, energy consumption of 3.6 kWh/day and water saving of 92% form a balanced model of economic and environmental management. At the same time, the environmental effect – a 40% reduction in catch from natural water bodies – confirmed that the system is not only economically advantageous, but also contributes to the conservation of biodiversity. Consequently, the use of the recirculating aquaculture system has the potential to become a key direction in the development of modern, environmentally oriented aquaculture business, which combines technological innovation, economic feasibility and nature-conservation effect. Such a system makes it possible not only to ensure stable production of high-quality hydrobiological products, but also to reduce pressure on natural water bodies, contributing to the gradual restoration. Thanks to minimal water consumption, low energy intensity, controlled microclimate and the possibility of multiple use of resources, the installation creates a closed ecological cycle within which all by-products of production can be repeatedly involved in the economic process.

DISCUSSION

The results obtained showed that the use of the recirculating water supply installation provided

stable conditions for the growth and development of river crayfish over the six months of the experiment. In the first week the juveniles were in the adaptation phase, but even at this stage one hundred per cent survival was recorded, which indicated the effectiveness of the conditions for the initial start-up of the system. The gradual increase in body length from 1.2 to 5.1 cm and in mass from 1.8 to 11.7 g demonstrated uniform growth without sharp fluctuations, which pointed to a balanced ratio between feed supply and the physicochemicalparameters of the environment. Overall, the temperature regime was maintained within 21-22°C, and the level of dissolved oxygen at 6.8-7.1 mg/l, which corresponded to the optimal conditions for the metabolism of *Astacus astacus*. Survival decreased only to 85% at the end of the experiment, which confirmed the minimal impact of stress factors and the absence of critical deviations in the operation of the system. The biofiltration complex effectively maintained chemical balance, reducing the concentration of ammonium nitrogen from 0.35 to 0.07 mg/l, nitrites from 0.15 to 0.03 mg/l, and nitrates from 4.8 to 2.8 mg/l. The parallel increase in pH to 7.5 and the rise in water transparency to 55 cm confirmed the stability of the functioning of the biocenosis of the filter. It was established that the formed self-regulating system maintained a closed nitrogen-exchange cycle without external intervention. This made it possible to minimise water consumption and to ensure a clean rearing environment. Crayfish cultivation reduced the pressure on natural populations and could significantly decrease the scale of poaching. With an increase in the volumes of legal rearing, the market price of crayfish decreased, which reduced the attractiveness of illegal harvesting.

Taking into account the influence of technological factors on the health of aquaculture organisms, the studies by L. Owens & J. Elliman (2025) and R. Wood *et al*. (2025) highlighted the issue of monitoring the status of crustacean and amphibian populations in altered environmental conditions. L. Owens & J. Elliman investigated the emergence of pathogens in rearing systems for *Cherax quadricarinatus* in Australia, establishing a relationship between holding conditions and the frequency of infectious outbreaks. R. Wood *et al*. applied environmental DNA methods to detect residual amphibian populations, demonstrating the advantages of molecular control. In contrast to these works, the present study did not require external genetic or microbiological intervention, since the stability of the recirculating water supply installations was maintained by natural biofiltration. In addition, the survival achieved at the level of 85-100% and the absence of pathogens confirmed the environmental safety and effectiveness of the closed purification cycle.

Considering the experience of studies of natural and artificial populations of aquatic invertebrates, the works of V.C. Terrell *et al*. (2023) and G. Kwikiriza *et al*. (2025) considered issues of population reproduction and the risks of aquaculture impacts on natural ecosystems. V.C. Terrell *et al*. established the dynamics of reproduction of *Rana areolata* under natural conditions, whereas G. Kwikiriza *et al*. emphasised the danger of escapes from aquaculture systems, which threatened the gene pool of local species. The present study demonstrated a different approach – a fully closed technological system without contact with the natural environment, which eliminated invasive risks. The experimental model provided stable population growth, a controlled hydrochemical regime and the possibility of completing the full life cycle without negative impacts on external aquatic ecosystems.

With regard to studies devoted to rare and protected crustacean species, the research of R.B. McCormack & N.S. Whiterod (2024) and C.C. Bloomer *et al.* (2024) examined the biological characteristics and conservation factors of indigenous species *Euastacus gamilaroi* and *prairie crayfish*. R.B. McCormack & N.S. Whiterod analysed threats to population degradation in natural waterbodies caused by disruption of the hydrological regime and human activity. C.C. Bloomer *et al*. noted that the application of conservation practices can contribute to stabilising population numbers, but these approaches did not provide for experimental control of environmental parameters. In comparison with these works, the present study was distinguished by experimental depth, clear regulation of hydrochemical indicators and practical orientation. Owing to the creation of a self-regulating recirculating water supply system, it was possible to achieve biological equilibrium, high survival and reproductive potential, which determines the prospects of this technology for the future restoration of natural populations.

Considering the results of studies devoted to the invasive potential of aquaculture species, the works of P.J. Haubrock *et al*. (2021) and A. Max-Aguilar *et al*. (2021) analysed the ecological risks and genetic diversity of *Cherax quadricarinatus*. P.J. Haubrock *et al*. determined that the expansion of cultivation of this species creates a threat to biodiversity in tropical regions due to its high adaptability and potential to escape into natural waterbodies. A. Max-Aguilar *et al.* assessed the genetic variability of populations in Mexico in order to create a breeding programme, but the study was limited to the population level without analysing the impact of holding conditions. The present study, on the contrary, was aimed at working out the technological parameters of keeping in a closed system, which completely eliminated ecological risks. The results obtained proved that stable indicators of growth, survival and hydrochemical balance can be achieved without the involvement of invasive species, providing an environmentally safe alternative to open systems.

Taking into account works oriented towards new monitoring methods and integrated rearing approaches, J.A. Greenhalgh *et al*. (2024) and Y. Wei *et al*. (2023) investigated various models combining environmental control and innovative technologies in aquaculture systems. J.A. Greenhalgh *et al*. showed that the use of environmental DNA can optimise species conservation strategies, but this approach remained exclusively diagnostic. Y. Wei *et al.* examined the Chinese integrated "rice-crayfish" system, which emphasised economic benefits, while at the same time noting difficulties with maintaining stable water quality. Unlike these works, the present study achieved a balance between technological stability and environmental sustainability: the closed system ensured stable hydrochemical parameters, consistent survival and minimal environmental impact, which makes it a more manageable and reproducible model.

Bearing in mind generalised data on intensification and physiological adaptation of crustaceans, the works of M.G. Emerenciano *et al*. (2022) and J.R. Latournerié-Cervera *et al*. (2025) considered various aspects of feeding, physiology and crustacean responses to environmental changes. M.G. Emerenciano *et al*. summarised advances in shrimp culture, emphasising the effectiveness of intensified systems, but without a detailed analysis of the environmental consequences. J.R. Latournerié-Cervera *et al*. examined the phenology of *Cambarellus montezumae* in Mexican waterbodies, revealing a relationship between water quality and population activity. In comparison with these works, the present study was of a more comprehensive nature, combining physiological, hydrochemical and biotechnical aspects. The stability achieved in growth indices, oxygen levels and water transparency confirmed that the presented recirculating water supply installation system can provide the complete life cycle of crayfish without ecological risks and without interference with natural populations.

Taking into account studies aimed at trends in fisheries, M. Eilitta *et al.* (2023) and G. Araujo *et al*. (2022) examined the ecological and technological challenges of modern aquaculture. M. Eilitta *et al*. analysed the population ecology of introduced crayfish in Zambia, establishing that invasive species adapt rapidly and displace local fauna, creating risks for natural biotopes. G. Araujo *et al*. summarised current trends in fish farming, emphasising the transition to technologically intensive systems, but without a detailed ecological assessment of the impact. Unlike these works, the present study focused on endogenous populations of *Astacus astacus* in a controlled environment without ecological risks. The achievement of stable growth indicators, high survival (85-100%) and a closed nitrogen cycle confirmed the advantage of the laboratory model over open or invasion-dependent systems.

Given the works devoted to spatial distribution and modelling of the spread of aquatic organisms, N.J. Mecha & R.G. Dolorosa (2024) and J.A. Daniels *et al.* (2023) studied environmental and infrastructural factors determining population dynamics in open waterbodies. N.J. Mecha & R.G. Dolorosa investigated larval collection sites of lobsters *Panulirus spp*. in the Philippines, identifying key hydrological parameters but without reproducing controlled growth conditions. J.A. Daniels *et al.* created a model of the spread of invasive species in river systems, focusing on the impact of hydraulic structures. In contrast to these works, the present study had an experimentally controlled character: all hydrochemical parameters were kept stable, which ensured the complete absence of migration risks and made it possible to trace growth patterns in an isolated biosystem.

Taking into account studies oriented towards biodiversity conservation and the digitalisation of aquaculture, M.V. Alvanou *et al.* (2022) and W.K. Cheng *et al*. (2025) described the latest approaches to monitoring crustacean populations. M.V. Alvanou *et al*. summarised the status of crayfish conservation in Greece, identifying problems of degradation of natural waterbodies and proposing measures for stock replenishment, but without developing technological solutions. W.K. Cheng *et al*. presented the concept of "smart aquaculture", in which the condition of crayfish was assessed by means of computer vision and Internet of Things systems. In comparison with these studies, the present experimental work had a practically verified character: hydrochemical stability, steady growth and minimal biomass losses showed that even without complex digital algorithms, the recirculating water supply installation system is capable of ensuring high efficiency, biosecurity, and reproducibility of results under real conditions. Thus, the results obtained demonstrated that stable water parameters, high survival and the gradual increase in crayfish biomass confirmed the effectiveness of the presented recirculating water supply system as an environmentally safe and technologically balanced aquaculture model.

CONCLUSIONS

In the course of the study, the effectiveness of the recirculating water supply installation for rearing the river crayfish *Astacus astacus* L. under laboratory-controlled conditions was experimentally confirmed. Over six months of rearing, the average length of the juveniles increased from 1.2 ± 0.1 cm to 5.1 ± 0.3 cm, and the average mass from 1.8 ± 0.3 g to 11.7 ± 0.5 g. Survival during this period remained high – from 100% at the beginning of the experiment to 85% at the end. Water temperature was maintained within 21-22°C, and the level of dissolved oxygen at 6.8-7.1 mg/l. Hydrochemical control showed a decrease in the concentration of ammonium nitrogen from 0.35 to 0.07 mg/l, nitrites from 0.15 to 0.03 mg/l and nitrates from 4.8 to 2.8 mg/l, with an increase in pH from 7.1 to 7.5 and an increase in transparency from 42 to 55 cm. This confirmed the effective functioning of the biofilter and the formation of a stable internal ecosystem. Behavioural analysis showed a clear positive trend: the activity of the juveniles increased from 2.1 ± 0.3 to 4.6 ± 0.1 points, the stress level decreased from 3.8 ± 0.4 to 1.8 ± 0.2 points, the proportion of cannibalism fell from 4.8% to 0.9%, and the reaction time to feed decreased from 38 to 15 seconds. This indicated stable metabolism and the formation of a socially coordinated behavioural structure of the population.

To assess adaptation under natural conditions, juveniles from the stage of the recirculating water supply installations were not used, but adult crayfish, selected separately as a control group of the adaptation block. For this reason, the average mass of individuals in the first month after release was 115 ± 4 g and was not related to the mass of the juveniles reared in the laboratory. After release into the waterbody, over six months survival gradually decreased from 100% to 72%, while the average mass increased from 115 ± 4 g to 133 ± 7 g. The activity of adult crayfish reached 4.6 ± 0.1 points, the stress level decreased to 1.7±0.1 points, and the proportion of females with eggs rose to 65%, which indicated complete restoration of reproductive function and stable physiological adaptation in the natural environment. Economic analysis showed that the cost price of rearing 1 kg of crayfish in the laboratory-production cycle was UAH 138, with an average market price of 310 UAH/kg. The expected net profit reached 2,050 UAH/m^2/year, and profitability exceeded 55%. The results obtained demonstrate that recirculating water supply technologies not only ensure stable growth and high survival, but also significantly reduce pressure on natural waterbodies, promoting the rational use of water resources. A limitation of the study was the relatively short observation period under natural conditions and the absence of comparative data for other crayfish species. Further research should be directed towards studying the reproductive cycle, optimising feeding schemes and developing scalable models of closed systems for industrial use.

ACKNOWLEDGEMENTS

None.

FUNDING

None.

CONFLICT OF INTEREST

None.

REFERENCES

[1] Agbugui, M.O., Inobeme, A., Okhamafe, E.P., Abhulimen, F.E., Abe, G., Odozie, J.C., & Ogbiti, J.T. (2025). Aquaculture production and management practices of farmed fish in Nigeria. In A. Gupta (Ed.), *Prospects of fungal biotechnologies for livestock: Fungal bioengineering in livestock health management* (volume 1, pp. 167-189). Cham: Springer. doi: 10.1007/978-3-031-90994-8_7.

[2] Agro-Ukraine. (n.d.). *Crayfish in the Kyiv region*. Retrieved from https://surl.li/niuneb.

[3] Alvanou, M.V., Papadopoulos, D.K., Lattos, A., Georgoulis, I., Feidantsis, K., Apostolidis, A.P., Michaelidis, B., & Giantsis, I.A. (2022). Biology, distribution, conservation status and stocking perspective of freshwater crayfish in Greece: An updated review. *Aquaculture Research*, 53(15), 5115-5128. doi: 10.1111/are.16009.

[4] Araujo, G.S., Silva, J.W, Cotas, J., & Pereira, L. (2022). Fish farming techniques: Current situation and trends. *Journal of Marine Science and Engineering*, 10(11), article number 1598. doi: 10.3390/jmse10111598.

[5] Bloomer, C.C., Miller, C.M., DiStefano, R.J., & Taylor, C.A. (2024). Midwest prairie management practices benefit the non-target prairie crayfish. *Fire Ecology*, 20, article number 11. doi: 10.1186/s42408-023-00243-x

[6] Cheng, W.K., Tan, T.B., San Tan, J., Yi-Le Chan, J., Bea, K.T., & Chen, Y.L. (2025). Smart aquaculture: Integrating computer vision and IoT for automated crayfish health assessment via federated learning. *IEEE Access*, 13, 189979-189994. doi: 10.1109/ACCESS.2025.3624140.

[7] Daniels, J.A., Kerr, J.R., & Kemp, P.S. (2023). River infrastructure and the spread of freshwater invasive species: Inferences from an experimentally-parameterised individual-based model. *Journal of Applied Ecology*, 60(6), 999-1009. doi: 10.1111/1365-2664.14387.

[8] DSTU EN ISO/IEC 17025:2019 "General requirements for the competence of testing and calibration laboratories". (2019). Retrieved from https://surl.li/xasewj.

[9] Duffy, R.E., Hodgson, B., Quinn, A., & Newman, M. (2025). Successful artificial incubation and juvenile-rearing of dropped eggs of a critically endangered freshwater crayfish (*Cherax tenuimanus*). *Marine and Freshwater Research*, 76(12), article number MF25063. doi: 10.1071/MF25063.

[10] Edwards, O.M., Banks, K.M., Zhang, B., & Reichert, M.S. (2025). Population dynamics and environmental drivers of crawfish frog (*Rana areolata*) migration in Southeastern Oklahoma, USA. *Herpetological Conservation Biology*, 20(2), 196-212.

[11] Eilitta, M., Nambeye-Kaonga, E., Gordon Mudenda, H., Katapa, C., Chimai-Mulenga, B., & Rice, A. (2023). *Population ecology and current distribution assessment of the introduced invasive crayfish in the Kafue Floodplain and Lake Kariba, Zambia*. Retrieved from https://surl.li/uaooxm.

[12] Emerenciano, M.G., Rombenso, A.N., Vieira, F.D., Martins, M.A., Coman, G.J., Truong, H.H., Noble, T.H., & Simon, C.J. (2022). Intensification of penaeid shrimp culture: An applied review of advances in production systems, nutrition and breeding. *Animals*, 12(3), article number 236. doi: 10.3390/ani12030236.

[13] European Commission. (2021). *Ethics and data protection*. Retrieved from https://surl.li/dyfdok.

[14] Greenhalgh, J.A., *et al*. (2024). Environmental DNA can inform the trade-off between proactive and reactive strategies for crayfish conservation. *Environmental DNA*, 6(3), article number e571. doi: 10.1002/edn3.571.

[15] Hapich, H., Novitskyi, R., Onopriienko, D., & Dubov, T. (2024). Water on fire: Losses and the post-war future of ecosystem services from water resources of Ukraine. *Regional Environmental Change*, 24, article number 154. doi: 10.1007/s10113-024-02320-6.

[16] Haubrock, P.J., Oficialdegui, F.J., Zeng, Y., Patoka, J., Yeo, D.C., & Kouba, A. (2021). The redclaw crayfish: A prominent aquaculture species with invasive potential in tropical and subtropical biodiversity hotspots. *Reviews in Aquaculture*, 13(3), 1488-1530. doi: 10.1111/raq.12531.

[17] Hinchcliffe, J., Agnalt, A.L., Daniels, C.L., Drengstig, A.R., Lund, I., McMinn, J., & Powell, A. (2022). European lobster *Homarus gammarus* aquaculture: Technical developments, opportunities and requirements. *Reviews in Aquaculture*, 14(2), 919-937. doi: 10.1111/raq.12634.

[18] Hrynevych, N., Zharchynska, V., Svitelskyi, M., Khomiak, O., & Sliusarenko, A. (2022). Promising object of aquaculture of crustaceans *Cherax quadricarinatus* (Von Martens, 1868): Biology, technology (review). *Aquatic Biological Resources and Aquaculture*, 1, 47-62. doi: 10.32851/wba.2022.1.4.

[19] Hudina, S., Maguire, I., Dragičević, P., & Galic, N. (2022). Evaluating the efficacy of approaches to control invasive populations: A conceptual model development for the signal crayfish. *Ecologies*, 3(2), 78-95. doi: 10.3390/ecologies3020008.

[20] Ishchuk, O., Svitelskyi, M., Matkovska, S., Sliusar, M., & Kovalchuk, I. (2024). Current status and development trends crustaceans aquaculture. *Ukrainian Journal of Natural Sciences*, 7, 18-24. doi: 10.32782/naturaljournal.7.2024.2.

[21] Kwikiriza, G., Muthoka, M., Omara, T., Abaho, I., Tibihika, P.D., Curto, M., Opiyo, M.A., Munguti, J., Abwao, J., Orina, P., & Meimberg, H. (2025). Nile tilapia (*Oreochromis niloticus* L.) cage aquaculture in Africa: Potential threats to congeneric fish species and advances to detect escapes. *Aquaculture, Fish and Fisheries*, 5(4), article number e70090. doi: 10.1002/aff2.70090.

[22] Latournerié-Cervera, J.R., Estrada-Ortega, A.R., Arana-Magallón, F., & Méndez-Ramírez, I. (2025). Ecophysiological studies of *Cambarellus montezumae* crayfish from Xochimilco, Mexico: Population phenology and water quality habitat. *European Journal of Biology and Biotechnology*, 6(2), 6-14. doi: 10.24018/ejbio.2025.6.2.537.

[23] Law of Ukraine No. 249 "On the Procedure for Carrying out Experiments and Experiments on Animals by Scientific Institutions". (2012, March). Retrieved from https://zakon.rada.gov.ua/laws/show/z0416-12#Text.

[24] Lee, S.S., Unger, A., Teo, S.L., & Shenkar, N. (2025). Culturing the solitary ascidian *Phallusia nigra* in closed and open water systems for tropical environmental research. *Limnology and Oceanography: Methods*, 23(10), 700-714. doi: 10.1002/lom3.10713.

[25] Max-Aguilar, A., Villarreal, H., Leyva-Valencia, I., Valencia-Valdez, R., Naranjo-Páramo, J., Vargas-Mendieta, M., Villarreal-Garcia, A., & Cruz-Hernández, P. (2021). Genetic diversity of divergent redclaw crayfish *Cherax quadricarinatus* (Von Martens, 1868) populations evaluated to initiate a breeding program in Mexico. *Latin American Journal of Aquatic Research*, 49(2), 272-279. doi: 10.3856/vol49-issue2-fulltext-2630.

[26] McCormack, R.B., & Whiterod, N.S. (2024). Biology, distribution, and conservation of the Gamilaroi crayfish *Euastacus gamilaroi* Morgan, 1997 (Decapoda: Astacidea: Parastacidae), a freshwater crayfish from New South Wales, Australia. *Journal of Crustacean Biology*, 44(1), article number ruae009. doi: 10.1093/jcbiol/ruae009.

[27] Mecha, N.J., & Dolorosa, R.G. (2024). Distribution and characteristics of spiny lobster (*Panulirus* spp.) puerulus collection sites in Palawan, the Philippines. *Philippine Journal of Science*, 153(3), 1061-1071. doi: 10.56899/153.03.26.

[28] Mohammed, R., Supain, C., Joseph, H., & Kahar, S.A. (2021). Freshwater lobster production-green aquaponics perennial system (FLP-GAPS): A green community-based social enterprise (CBSE) for B40 in Sabah. In R. Hassan, N.H. Abdul Hamid, A.K. Arshad, A. Alisibramulisi, M. Norhasri Muhd Sidek, N. Mohamad Bhkari & E. Shaffie (Eds.), *Green infrastructure: Materials and applications* (pp. 285-304). Singapore: Springer. doi: 10.1007/978-981-16-6383-3_17.

[29] Oficialdegui, F.J., *et al*. (2025). Contrasting patterns of genetic variability in pet-traded red swamp crayfish *Procambarus clarkii* and its feral populations. *Freshwater Biology*, 70(2), article number e70008. doi: 10.1111/fwb.70008.

[30] Owens, L., & Elliman, J. (2025). Effect of husbandry on the emergence of pathogens in the aquaculture of redclaw crayfish (*Cherax quadricarinatus*) in Australia. *Aquaculture*, 603, article number 742380. doi: 10.1016/j.aquaculture.2025.742380.

[31] Qin, L., Guo, C., Xiong, M., Gong, K., Liu, J., Zhang, T., & Li, W. (2023). Effects of shelter on the hatching, immune performance, and profitability of the Ovigerous Red Swamp Crayfish *Procambarus clarkii* under High Stocking Density. *Water*, 15(5), article number 907. doi: n10.3390/w15050907.

[32] State Agency for Land Reclamation, Fisheries, and Food Programmes of Ukraine. (n.d.). *Industrial fishing of aquatic biological resources*. Retrieved from https://surl.li/uzjglf.
[33] Terrell, V.C., Maerz, J.C., Engbrecht, N.J., Stiles, R.M., Crawford, B.A., & Lannoo, M.J. (2023). Breeding population dynamics of threatened crawfish frogs inform targets for habitat management. *Ichthyology & Herpetology*, 111(1), 72-86. doi: 10.1643/h2022031.
[34] Wei, Y., Müller, D., Sun, Z., Lu, M., Tang, H., Wu, W. (2023). Exploring the emergence and changing dynamics of a new integrated rice-crawfish farming system in China. *Environmental Research Letters*, 18(6), article number 064040. doi: 10.1088/1748-9326/acd8d2.
[35] Wood, R., Coleman, D., McFadden, M., Spark, P., Coote, D., Hill, P., & Furlan, E. (2025). Applying eDNA methods to monitor the distribution of remnant and translocated populations of an endangered amphibian, *Litoria booroolongensis*. *Environmental DNA*, 7(5), article number e70208. doi: 10.1002/edn3.70208.

Екологічна ефективність установок замкнутого водопостачання при штучному розведенні річкового раку

Артем Кравченко

Аспірант
Дніпровський державний технічний університет
51918, вул. Дніпробудівська, 2, м. Кам'янське, Україна
https://orcid.org/0009-0004-7848-6239

Катерина Божко

Кандидат біологічних наук, доцент
Дніпровський державний технічний університет
51918, вул. Дніпробудівська, 2, м. Кам'янське, Україна
https://orcid.org/0000-0002-1481-7164

Анотація. Метою дослідження було оцінити ефективність технології замкненого водного циклу, що знижує вплив на природні водойми та сприяє раціональному використанню водних ресурсів. У дослідженні застосовувалися експериментальні, аналітичні та статистичні методи: біометричні вимірювання, гідрохімічний контроль, поведінкові спостереження й кореляційний аналіз. На початку експерименту середня довжина малька становила 1,2 см, маса – 1,8 г, тоді як наприкінці спостережень показники зросли до 5,1 см і 11,7 г відповідно. Виживаність зберігалася у межах 85-100 %, що свідчило про оптимальні умови культивування. Температура води підтримувалася стабільно на рівні 21-22°C, концентрація кисню коливалася в межах 6,8-7,1 мг/л, забезпечуючи нормальний метаболізм гідробіонтів. Гідрохімічний аналіз показав ефективну роботу біофільтра: концентрація амонійного азоту зменшилася з 0,35 до 0,07 мг/л, нітритів – з 0,15 до 0,03 мг/л, а нітратів – з 4,8 до 2,8 мг/л. Прозорість води зросла з 42 до 55 см, а pH стабілізувався на рівні 7,5, що свідчило про завершення етапу біологічної стабілізації середовища. Поведінкові показники також підтвердили успішну адаптацію: активність молоді збільшилася з 2,1 до 4,6 балів, рівень стресу знизився з 3,8 до 1,8 балів, а канібалізм скоротився до 0,9 %.Після переселення у природну водойму виживаність раків становила 72 %, середня маса – 133 г, а частка самиць з ікрою досягла 65 %, що підтвердило природну здатність до відтворення. Економічні розрахунки засвідчили рентабельність виробництва 55,2 % при собівартості 138 грн/кг та чистому прибутку 2050 грн/м^2/рік. Енергоспоживання системи було низьким – 3,6 кВт·год/добу, економія водних ресурсів – 92 %, екологічний ефект – зниження вилову природних популяцій на 40 %. Практична значимість даного дослідження полягає у можливості використання його результатів для впровадження енергоефективних технологій вирощування річкових раків у фермерських та промислових господарствах України

Ключові слова: природні популяції; виживаність; гідробіонти; рентабельність; адаптація

Received: 30.05.2025 Revised: 21.10.2025 Accepted: 27.11.2025 Published: 26.12.2025

BIOLOGICAL SYSTEMS: Theory and Innovation

ISSN 2706-8382
e-ISSN 2706-8390

Journal homepage: https://bioscience.com.ua/en
Vol. 16, No. 4, 2025, 40-56

UDC 639.9:582.581:602.6

DOI: 10.31548/biologiya/4.2025.40

Microclonal propagation of the orchid *Vanda* (*Orchidaceae Vanda*)

Oksana Kliachenko*

Doctor of Agricultural Sciences, Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
https://orcid.org/0000-0002-4087-4082

Oleksandr Subin

PhD in Biological Sciences, Associate Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
https://orcid.org/0000-0001-6209-2439

Olha Oliinyk

PhD in Agricultural Sciences
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
https://orcid.org/0000-0002-3591-3518

Diana Shyshkina

Student
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
https://orcid.org/0009-0001-2486-7691

Abstract. Biotechnological methods of propagation of *Vanda* orchids represent an effective approach for obtaining large quantities of genetically uniform and disease-free plant material. This study aimed to investigate the specific features of microclonal propagation of *Vanda* orchids. To obtain planting material of different *Vanda* genotypes, namely 'Vanda coerulea' and 'Vanda sanderiana', direct and indirect morphogenesis, as well as *in vitro* rhizogenesis followed by adaptation to *in vivo* conditions, were applied. A stepwise method for obtaining aseptic material was developed, involving treatment with 70% ethyl alcohol for 1 minute followed by the application of the main sterilising agent, 2.5% NaClO, for 5-10 minutes. This protocol made it possible to obtain 70-80% sterile and viable explants while reducing the level of fungal and bacterial contamination. The results of direct and indirect morphogenesis, callus formation, and rhizogenesis in *in vitro* cultures of *Vanda* explants are presented. No significant differences in callus formation were observed between the studied cultivars. The frequency of callusogenesis for both genotypes reached 100%, indicating a high capacity for callus tissue formation. The best results in growth and shoot formation were achieved on Murashige and Skoog (MS) nutrient medium supplemented with 0.5 mg/L 6-benzylaminopurine (6-BAP). For rooting, the most effective medium was MS with halfstrength macro- and microelements supplemented with 0.5 mg/L α-naphthaleneacetic acid (NAA). This medium is recommended for the induction of rhizogenesis in regenerated plants of different

Suggested Citation:

Kliachenko, O., Subin, O., Oliinyk, O., & Shyshkina, D. (2025). Microclonal propagation of the orchid *Vanda* (*Orchidaceae Vanda*). *Biological Systems: Theory and Innovation*, 16(4), 40-56. doi: 10.31548/biologiya/4.2025.40.

*Corresponding author

Vanda cultivars. For the adaptation of regenerated plants, a substrate composed of sphagnum moss, peat, crushed beech leaves, and pine bark in a ratio of 1:1:1:1 was used. The survival rate of 'Vanda coerulea' plants under *in vivo* conditions reached 87.5%, whereas under the same conditions, the survival rate of 'Vanda sanderiana' plants was 83%. The obtained results may serve as a basis for the development of propagation protocols for *Vanda* orchids, ensuring a high level of control at all stages of cultivation. The implementation of such protocols is important for breeding programmes, the conservation of rare cultivars, and the improvement of plant material quality for commercial floriculture

Keywords: *in vitro*; callusogenesis; morphogenesis; rhizogenesis; *in vivo* adaptation

INTRODUCTION

Orchids of the genus *Vanda* are among the most striking representatives of the family *Orchidaceae*, distinguished by their high ornamental value, diversity of morphological forms, and long-lasting, intense flowering. Owing to these characteristics, they have gained popularity among amateur and professional growers not only worldwide but also in Ukraine. These plants are epiphytes or lithophytes native to tropical regions, naturally occurring in South-East Asia, the Philippines, Indonesia, northern Australia, and the Himalayas. The particular interest in *Vanda* orchids is due not only to their aesthetic appeal but also to their high commercial value, which makes them an important object for ornamental horticulture and large-scale propagation. However, traditional methods of propagation, such as seed propagation and vegetative reproduction, have significant limitations, including a long developmental cycle and an increased risk of viral infection, which restricts their efficiency for industrial cultivation. Therefore, the development of modern biotechnological approaches for the propagation of *Vanda* orchids is highly relevant. In particular, microclonal propagation makes it possible to obtain large quantities of genetically uniform and virus-free plant material, which in turn contributes to biodiversity conservation, improves cultivation efficiency, and ensures a stable supply of high-quality planting material. The study of biotechnological characteristics of orchids of the genus *Vanda* and the improvement of methods for their microclonal propagation represent key directions in the development of modern plant science, biotechnology, and the conservation of ornamental flora (Liu *et al.*, 2020; Pathak *et al.*, 2023).

One of the important research directions highlighted in the literature is the optimisation of *in vitro* culture conditions to enhance the efficiency of microclonal propagation of *Vanda* orchids. K. Nowakowska *et al*. (2022) demonstrated that the efficiency of microclonal propagation of *Vanda brunnea* Rchb.f. primarily depends on the mineral composition of the nutrient medium and is not influenced by the addition of 0.5 mg/L 6-BAP. L. Tikendra *et al*. (2025) reported that the highest multiplication coefficient (5.62) was achieved on MS medium supplemented with 1.2 mg/L kinetin and 0.6 mg/L indole-3-butyric acid. In the studies by X.-f. Liu *et al*. (2020) and P. Pathak *et al*. (2023), the effects of plant growth regulators on seed germination and protocorm formation in orchids were investigated. X.-f. Liu *et al*. (2020) emphasised the effective use of growth regulators to stimulate seed germination of *Vanda falcata*, which significantly enhances the success of *in vitro* propagation of this species. At the same time, P. Pathak *et al*. (2023) examined the influence of similar compounds on vegetative propagation and the phytochemical composition of *Vanda cristata*, an endangered orchid species. The investigation of these aspects is of considerable importance for the biopharmaceutical industry. A.N. Laily *et al*. (2024) studied the effect of organic components on the rate of protocorm formation and their viability. Another important aspect is the maintenance of the genetic uniformity of regenerated plants during *in vitro* cultivation. In the study by R. Baby *et al*. (2019a), it was determined that, using RAPD, SSR, and ISSR markers, the level of genetic polymorphism in regenerated plants did not exceed 5% regardless of the duration of cultivation; however, this value increas ed after six subcultures.

It should be noted that the natural propagation of *Vanda* orchids occurs through seed reproduction, which is a highly complex process because the seeds lack endosperm and therefore cannot

germinate independently. Seed germination is possible only in the presence of mycorrhizal fungi, which supply the embryo with essential nutrients. This characteristic is typical of all orchids and makes natural seed-based reproduction an expensive and relatively unreliable method. H.N. Rasmussen & F.N. Rasmussen (2014) examined the evolution of mycorrhiza in orchids, which is essential for understanding their development, particularly in the context of microclonal propagation. Mycorrhiza plays a key role in seed germination and seedling development, thereby confirming the effectiveness of *in vitro* cultivation methods for orchids. B.J. Davis *et al*. (2015) investigated the distribution of mycorrhizal fungi, which is important for the biogeography of orchids, especially with regard to their rooting and development. The study by A. Setiaji *et al*. (2021) demonstrated the effectiveness of biotechnological methods of microclonal propagation of *Vanda* orchids, making it possible to obtain genetically uniform and disease-free planting material. The most effective approach involved the use of Murashige and Skoog medium supplemented with 6-BAP for growth and shoot formation, as well as media supplemented with α-naphthaleneacetic acid for rooting. The developed sterilisation and adaptation methods ensured high plant survival rates and can be applied in industrial orchid production.

In this context, microclonal reproduction techniques, such as *in vitro* meristem culture, have become extremely popular in ornamental horticulture. These biotechnological approaches enable the production of large numbers of genetically identical, healthy plants that retain the morphological and decorative characteristics of the parent plant. This is of critical importance both for the conservation of rare cultivars and for their large-scale commercial propagation. This study aimed to investigate the specific features of microclonal propagation of *Vanda* orchids and to obtain healthy, virus-free planting material under *in vitro* conditions.

MATERIALS AND METHODS

The research was conducted in the Biotechnology and Bioengineering Educational and Research Laboratory of the NULES of Ukraine from 2023 to early 2025. The orchid cultivars 'Vanda coerulea' and 'Vanda sanderiana', characterised by high ornamental value and long-lasting flowering, were used for introduction into *in vitro* culture. Generally accepted biotechnological research methods were applied (Kolomiets *et al*., 2022). The study was carried out in several directions: investigation of the callusogenic capacity of *Vanda* orchids, analysis of direct and indirect morphogenesis, assessment of shoot formation intensity under *in vitro* conditions, rooting of regenerated plants, and their adaptation to *in vivo* conditions.

For the experiments, explants were collected from healthy donor plants. Before introduction into sterile culture, particular attention was paid to the sterilisation stage in order to prevent fungal and bacterial contamination during subsequent cultivation. Initially, the explants were thoroughly washed in a soap solution for 10-15 minutes to remove surface contaminants. They were then rinsed with running tap water, followed by distilled water, and finally twice with sterile distilled water. All subsequent procedures were performed exclusively under a laminar airflow cabinet in strict compliance with aseptic techniques. Protocorms and young flower shoots not exceeding 2 cm in length were used as primary explants.

Sterilisation was carried out using stepwise treatment of the explants according to the following schemes: 70% ethyl alcohol (1-2 min) → 2.5% NaClO (exposure for 5, 10, or 15 min); 70% ethyl alcohol (1-2 min) → 1% AgNO3 (exposure for 5, 10, or 15 min); 70% ethyl alcohol (12 min) → 2.5% NaClO + 1% AgNO3 (15 min). The sterile explants were rinsed three times for 10 minutes each with sterile distilled water. Afterwards, they were placed in Petri dishes on sterile filter paper for drying. Using sterile forceps and observing aseptic conditions, the explants were transferred into test tubes containing hormone-free Murashige and Skoog (MS) nutrient medium (Murashige & Skoog, 1962) and cultured in a thermostat in the absence of light at a temperature of 25°C ± 1°C. On the seventh day after the initiation of the experiment, the cultures were examined for the presence of microbiological contamination. Infected samples were removed from the thermostat, as they represented a potential source of infection. The sterilisation efficiency (Es), expressed as a percentage, was calculated using the following formula:

$$Es = \frac{Ne - Ndme}{Ne} \times 100\%, \quad (1)$$

where N_e is the total number of planted explants (units), and N_{dme} is the number of damaged (infected) explants (units). For callus induction, the explants were transferred to nutrient media MSK1–MSK3 supplemented with α-naphthaleneacetic acid (NAA) at concentrations of 2-5 mg/L and 0.2 mg/L 6-benzylaminopurine (6-BAP), as well as 100 mg/L myo-inositol, 500 mg/L casein hydrolysate, and 30 g/L sucrose. For subculturing of *Vanda* orchid callus tissue, the average fresh weight of the inoculum was 2.0 ± 0.10 g. The frequency of callus formation was determined as the percentage ratio of explants that successfully formed callus to the total number of samples. The average monthly increase in callus fresh weight was calculated as the difference between the final mass after cultivation and the initial mass placed on the nutrient medium, in accordance with the methodology described by Yu.V. Kolomiets *et al*. (2022).

For the induction of indirect morphogenesis, callus tissue of *Vanda* orchids with an average mass of 2.0 ± 0.10 g was subcultured onto MSH1–MSH3 nutrient media enriched with phytohormones: 6-BAP at concentrations of 0.1-3.0 mg/L, kinetin at 0.5-2.0 mg/L, and 2,4 dichlorophenoxyacetic acid (2,4-D) in the range of 0.1-1.0 mg/L. Cultivation was carried out under controlled conditions at a temperature of 25°C ± 1°C, light intensity of 2.0-3.0 klx, a 16-hour photoperiod, and relative humidity of 70-75%. At the shoot formation stage, apical shoot segments and microcuttings 1-2 cm in length, containing a pair of leaves and part of the aerial roots where meristematic tissues are located, were used, as these tissues possess a high capacity for new shoot formation. Owing to the appropriate selection of the phytohormonal composition, particularly the balanced combination of cytokinins and auxins, positive results were achieved, whereby several new plants were obtained from a single explant. Subsequently, rhizogenesis was stimulated, taking into account that a well-developed root system ensures plant survival after transplantation to *in vivo* conditions. For this purpose, regenerated shoots were transferred to a medium with a reduced content of macronutrients supplemented with auxins. Indole-3-acetic acid (IAA) and indole3butyric acid (IBA) were most frequently applied at concentrations of 0.5-2.0 mg/L. These conditions promoted the formation of root primordia within the first two weeks of cultivation, which is consistent with the recommendations reported by T.D. Thomas (2008), B. Bhattacharjee & S.M.S. Islam (2014b), and A. Setiaji *et al*. (2021).

At the final stage, the orchid plants were adapted to *in vivo* conditions by transplanting them into a moist, well-drained substrate composed of sphagnum moss, peat, crushed beech leaves, and pine bark in a ratio of 1:1:1:1, followed by placement under conditions of high humidity. The cultivation temperature was maintained within the range of 23°C-25°C, and irrigation was carried out only after partial drying of the upper layer of the substrate. The experimental data obtained during the study were subjected to statistical analysis using the MS Excel software package. All experiments were performed in triplicate, and 30 explants were used in each experimental variant. The tables present arithmetic mean values and their standard errors. Experimental studies involving plants, including the collection of plant material, complied with institutional, national, and international guidelines. The authors adhered to the standards of the Convention on Biological Diversity (1992).

RESULTS AND DISCUSSION

Selection, preparation of explants, and introduction into *in vitro* culture for obtaining aseptic explants of *Vanda* orchids

The stage of explant selection is one of the most critical steps in the process of microclonal propagation, as the quality of the initial material largely determines the subsequent success of cultivation. Various plant parts may be used as explants, including apical or axillary buds, stem fragments, leaves, aerial roots, or meristematic tissues, each of which has specific characteristics. The choice of explant type directly influences the efficiency of the process (Chugh *et al.*, 2009; Bhattacharjee & Islam, 2014a). For *Vanda* orchids, the use of young, non-lignified aerial roots or apical buds is considered appropriate, as these tissues exhibit high regenerative capacity and are less susceptible to pathogen infection. In addition, the physiological condition of the plant and the environmental conditions under which the material is collected are of great importance (Rahman *et al.*, 2009; Nowakowska *et al.*, 2022; Tikendra *et al.*, 2025).

A necessary prerequisite for the successful application of microclonal propagation methods is strict adherence to specific rules. First of all, the tissues intended for cultivation must be aseptically isolated from the parent plant. Obtaining sterile material for cultivation is a challenging task, as the plant surface is naturally colonised by epiphytic bacteria, fungi, and their spores. The correct selection of sterilising agents aims to neutralise the epiphytic microflora without damaging plant tissues. In addition, the sterilising substance should not penetrate deeply into the tissues and must be easily removed by washing. To obtain an aseptic culture of *Vanda* orchids, visually healthy, morphologically normal plants without external symptoms of bacterial, fungal, or viral infection were selected. The plants were thoroughly washed under running water to remove surface contaminants from the inflorescences, aerial roots, and leaves. To prevent microbial contamination, subsequent sterilisation procedures were performed in a laminar airflow cabinet.

The sterilisation methodology was selected experimentally for each object, depending on tissue sensitivity. Furthermore, the same organ from different plants requires different sterilisation conditions. Therefore, to achieve the research objectives, several commonly used sterilising agents with varying exposure times were applied. The sterilisation variants for explants of 'Vanda coerulea' and 'Vanda sanderiana', and the corresponding results are presented in Tables 1 and 2. For cultivation, the basal Murashige and Skoog nutrient medium was used. Following sterilisation, the explants were cultured on hormone-free MS medium at a temperature of 25°C ± 1°C, relative humidity of 70-80%, and a 16-hour photoperiod.

Table 1. Sterilisation efficiency of 'Vanda coerulea' explants

No.	Sterilising agent	Concentration (%)	Exposure (min)	Number of explants introduced *in vitro* (pcs)	Number of aseptic explants (pcs)	Number of viable explants (pcs)	Sterilisation efficiency (%)*
1	NaClO	2.5	5	30	26	24	80
2	NaClO	2.5	10	30	28	21	70
3	NaClO	2.5	15	30	30	10	33
4	$AgNO_3$	1	5	30	17	15	50
5	$AgNO_3$	1	10	30	25	18	60
6	NaClO $AgNO_3$	2.5 1	15 15	30	30	3	10
LSD_{05}					1.3	0.8	2.5

Note: * – calculated using formula 1
Source: developed by the authors

It should be noted that fungal contamination of 'Vanda coerulea' explants was observed on days 8-10, whereas bacterial and mixed fungal-bacterial contamination occurred between days 5 and 15 of cultivation. The experimental data indicate that the most effective method for sterilising explants was the stepwise treatment: 70% ethyl alcohol (1 min) → 2.5% NaClO (5-10 min), which allowed the production of 70-80% sterile and viable explants (Fig. 1). Overall, a positive outcome (~50-60% aseptic and viable explants) was obtained using 1% $AgNO_3$ for 5-10 minutes. However, the use of sterilising agents such as 2.5% NaClO and 1% $AgNO_3$ with a 15-minute exposure negatively affected explant viability due to tissue oxidation, and the sterilisation efficiency decreased to 10%.

The results of sterilising the inflorescences of 'Vanda sanderiana' are presented in Table 2.

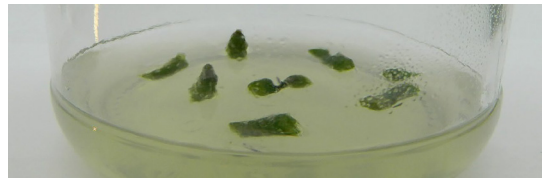

Figure 1. Aseptic protocorms of 'Vanda coerulea' (sterilisation variant No. 1)
Source: provided by the authors

Table 2. Sterilisation efficiency of 'Vanda sanderiana' explants

No.	Sterilising agent	Concentration (%)	Exposure (min)	Number of explants introduced *in vitro* (pcs)	Number of aseptic explants (pcs)	Number of viable explants (pcs)	Sterilisation efficiency (%)*
1	NaClO	2.5	5	30	26	25	80
2	NaClO	2.5	10	30	28	20	70
3	NaClO	2.5	15	30	30	9	31
4	$AgNO_3$	1	5	30	15	13	45
5	$AgNO_3$	1	10	30	25	18	60
6	NaClO $AgNO_3$	2.5 1	15 15	30	30	3	10
LSD_{05}					1.3	0.7	2.5

Note: * – calculated using formula 1
Source: developed by the authors

From the data presented in Table 2, it can be seen that, for 'Vanda sanderiana' explants, the most effective sterilisation method, as with 'Vanda coerulea', was the stepwise approach: treatment with 70% ethyl alcohol for 1 minute followed by immersion in 2.5% NaClO for 5-10 minutes. This protocol allowed the production of 70-80% sterile and viable explants (Fig. 2). This sterilisation regime did not damage the tissues or inhibit explant development, while ensuring maximal sterility. A positive result (45-60% aseptic and viable explants) was also obtained using 1% $AgNO_3$ for 510 minutes. Exposure to 2.5% NaClO or 1% $AgNO_3$ for 15 minutes adversely affected viability due to tissue oxidation. The lowest number of aseptic and viable explants was observed under sterilisation variant No. 4.

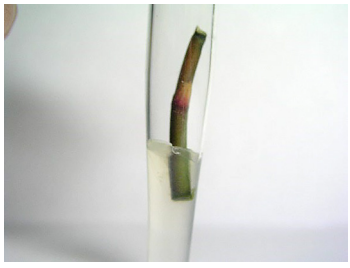

Figure 2. Aseptic and viable explants of 'Vanda coerulea' on day 30 of cultivation (sterilisation variant No. 1)
Source: provided by the authors

There is a trend towards minimising the use of highly toxic compounds as sterilising agents. In the study by R. Baby *et al*. (2019b), a stepwise method was employed for sterilising *Vanda* orchid explants using 0.1% $HgCl_2$ as the primary sterilant with exposure times ranging from 1 to 10 minutes. The percentage of sterile explants obtained varied from 0% to 80%. With longer exposure, the toxic effect of 0.1% $HgCl_2$ on explant tissues became apparent. Therefore, the approach used in the present study, employing 2.5% NaClO, is both safer and sufficiently effective for working with inflorescence shoots and protocorms. As a result, aseptic and viable explants of 'Vanda coerulea' and 'Vanda sanderiana' were obtained and subsequently used in studies on callusogenesis, morphogenesis, and plant regeneration.

Features of callusogenesis in *Vanda* orchids

In higher plants, callus tissue is defined as tissue formed through uncontrolled cell proliferation in explants following their initial subculture. This process is widely applied in biotechnological research as well as in microclonal propagation of plants. Studies have shown that the process of callusogenesis *in vitro* largely depends on the external culture conditions and the composition of the nutrient medium, particularly the presence of plant growth regulators. Effective callus induction is generally observed when using the classical auxin-to-cytokinin ratio of 10:1 (Kushnir & Sarnavska, 2005). It should be noted that plant growth regulators play a key role in stimulating cell proliferation and structural changes within tissues, thereby enabling the formation of callus tissue with high potential for subsequent regeneration. In *Vanda* orchids, callus tissue is typically induced from protocorms and inflorescence shoots by culturing them on modified MS

medium supplemented with growth regulators, specifically α-naphthaleneacetic acid and 6 benzylaminopurine, at various concentrations. Three variants of nutrient media were used for callus induction (Table 3).

Explants were placed in sterile flasks containing the nutrient medium and cultured in the dark at 25°C ± 1°C for 14 days. An increase in explant size was observed, indicating active uptake of carbohydrates and mineral nutrients from the medium. Subsequently, the explants were transferred to a light regime with an intensity of 3,000 lx and a 16-hour photoperiod, and cultured at 24°C ± 1°C. On day 30, the frequency of callus formation and the biomass of the callus were recorded, with the results presented in Table 4.

Table 3. Growth regulator composition in callus-inducing media

Variant	Growth regulators
1	2 mg/L NAA + 0.2 mg/L 6-BAP
2	5 mg/L NAA + 0.2 mg/L 6-BAP
3	2.5 mg/L NAA + 0.4 mg/L 6-BAP

Source: developed by the authors

Table 4. Dependence of callusogenesis in *Vanda* orchids on the content of growth regulators in the nutrient medium

Variant	Medium composition	Number of explants (pcs)	Number of explants forming callus	
			(%)	Callus fresh weight (mg)
'Vanda coerulea'				
1	2 mg/L NAA + 0.2 mg/L 6-BAP	30	90 ± 4.5	270 ± 13.5
2	5 mg/L NAA + 0.2 mg/L 6-BAP	30	97.5 ± 4.9	540 ± 24.3
3	2.5 mg/L NAA + 0.4 mg/L 6-BAP	30	100 ± 5.0	690 ± 30.2
'Vanda sanderiana'				
1	2 mg/L NAA + 0.2 mg/L 6-BAP	30	88 ± 4.4	300 ± 15.0
2	5 mg/L NAA + 0.2 mg/L 6-BAP	30	95 ± 4.8	490 ± 24.5
3	2.5 mg/L NAA + 0.4 mg/L 6-BAP	30	99 ± 4.9	655 ± 32.8

Source: developed by the authors

From the data presented, it can be seen that the highest callus mass and the greatest percentage of callus formation were recorded on MS medium variant 3, where the concentrations of NAA and 6-BAP were 2.5 mg/L and 0.4 mg/L, respectively. This high regenerative capacity aligns with the findings of A.N. Laily *et al*. (2024), who reported that the correct auxin-to-cytokinin ratio is critical for callus induction in *Vanda celebica*. In general, the optimal ratio of auxins to cytokinins for callus formation is quite variable and depends on the specific culture. In the study by S.A. Dar *et al*. (2021), callus induction in *Atropa acuminata* was most effective at a 1:1 ratio of 6BAP to NAA, while A.F. Likhanov *et al*. (2017) observed callus induction in *Aesculus hippocastanum* using kinetin and 2,4-D at a 1:6 ratio. A similar trend is generally observed across the *Orchidaceae* family (Sheyko & Musatenko, 2013; Shemediuk & Skip, 2014). In addition to quantitative characteristics, the density and colour of the callus tissue were also assessed. In *Vanda* orchids, the callus was typically cream-white or yellowish in colour, with a loose to medium texture and pronounced meristematic activity, indicating a high regenerative potential of the tissue (Fig. 3).

Figure 3. Callus tissue of 'Vanda coerulea'
Source: provided by the authors

Thus, according to the results of the conducted studies, the most effective callus-inducing medium for both *Vanda* orchid cultivars was MS medium supplemented with 2.5 mg/L NAA and 0.4 mg/L 6-BAP. Under these conditions, 99-100% of the explants produced callus, demonstrating the high efficiency of this medium for inducing callusogenesis. The fresh callus mass obtained on this medium was 690 mg for 'Vanda coerulea' and 655 mg for 'Vanda sanderiana'.

Features of indirect morphogenesis in *Vanda* orchids

One of the least explored aspects of cellular differentiation is morphogenesis. The main challenge in studying the physiological, biochemical, and molecular processes underlying it lies in the asynchrony of cell differentiation within tissues that later form organs. Several researchers have shown that morphogenetic centres are spatially distinct, and the cells involved exhibit varying levels of metabolic activity. During the cultivation of unorganised callus tissue, diverse morphological structures arise, eventually forming shoots, roots, leaves, and in some cases, complete plants (Palama *et al*., 2010; Lee *et al*., 2013; Hardjo *et al*., 2021). In Vanda orchids, indirect morphogenesis typically involves two main stages: first, callus tissue is formed from the explant, and subsequently, morphogenetic structures develop within the callus, either through organogenesis or somatic embryogenesis. The formation of organs within the callus culture is closely associated with the ratio of plant growth regulators.

Callus tissues of *Vanda* orchids exhibit the capacity for both somatic embryogenesis and organogenesis, leading to the formation of shoots, roots, and even floral structures. This developmental plasticity makes the genus highly promising for large-scale microclonal propagation. At the same time, the potential for organogenesis is higher in tissues closer to the plant base, whereas explants isolated from the stem show reduced morphogenetic activity. Furthermore, repeated subculturing of callus cultures gradually diminishes the capacity to form new organs, although root formation may remain stable over an extended period (Lee *et al*., 2013; Chen, 2018; Hardjo *et al*., 2021). In addition, studies by R. Baby *et al*. (2019a) and K. Nowakowska *et al*. (2022) have shown that callusogenesis carries a risk of somaclonal variation. This is particularly important for rare and endangered species, where maintaining the genotype is critical; thus, the use of molecular markers to monitor regenerant plants is necessary.

To stimulate indirect morphogenesis, callus tissues with a mass of 2.0 ± 0.10 g were cultured on modified MS medium supplemented with 6-benzylaminopurine, kinetin, and 2,4 dichlorophenoxyacetic acid at varying concentrations. Explants were maintained at 25°C ± 1°C under a light intensity of 2.0-3.0 klx, a 16-hour photoperiod, and 75-80% relative humidity. During the study, active organogenesis was observed in both Vanda cultivars under investigation (Table 5).

Table 5. Organogenic potential *in vitro* of different *Vanda* orchid cultivars

Passage No.	Organogenesis intensity		
	Organogenesis frequency (%)	Number of shoots (pcs)	Shoot height (cm)
'Vanda coerulea'			
1-3	100 ± 5	4.0 ± 0.25	7.50 ± 0.10
5-6	55 ± 2.8	2.1 ± 0.18	5.20 ± 0.14
7-8	13 ± 0.7	0.4 ± 0.07	2.10 ± 0.09
'Vanda sanderiana'			
1-3	98 ± 4.9	5.5 ± 0.3	7.0 ± 0.4
5-6	65 ± 3.3	3.2 ± 0.2	6.1 ± 0.3
7-8	15 ± 0.8	18 ± 0.9	2.8 ± 0.1

Source: developed by the authors

The results of the study indicate that the most intensive organogenesis in Vanda orchid cultivars occurs during passages 1-3 (each passage lasting 21 days) (Fig. 4). However, with further increases in the number of passages (beyond 5-6), shoot formation activity declined significantly and almost

ceased after passages 8-9. This reduction is likely attributable to somaclonal variation arising from prolonged cultivation.

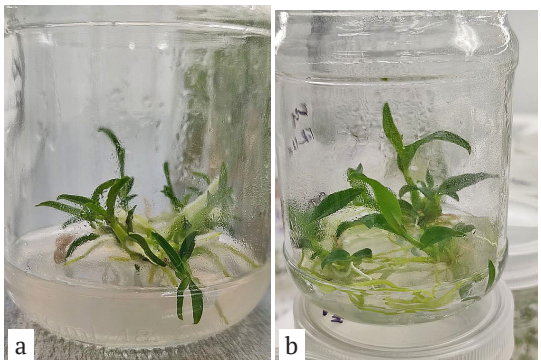

Figure 4. Shoots of 'Vanda coerulea' derived from callus tissue
Note: a – passages 5-6; b – passages 1-3
Source: provided by the authors

Thus, the study established that the efficiency of indirect morphogenesis in *Vanda* orchids depends on several factors, namely the genotype of the donor plant, the presence of growth regulators in the culture medium, and the number of callus subcultures. To maintain high morphogenetic activity, it is recommended to limit the number of passages to 5-6 and periodically renew the original callus. Induction of direct morphogenesis in orchid genotypes. Apical meristems are a key source of explants in the microclonal propagation of orchids, particularly in the genus *Vanda*. Their high meristematic activity and capacity to form secondary shoots under appropriate culture conditions highlight the importance of this type of explant material (Lang & Hang, 2006; Rahman *et al.*, 2009).

One of the decisive factors for successful plant regeneration is the carefully selected composition of the culture medium, particularly the optimal concentration of plant growth regulators. The interaction between cytokinins and auxins plays a crucial role in regulating morphogenesis. Research has demonstrated that the combination of 6-benzylaminopurine (6-BAP) with a low concentration of IAA promotes the intensive formation of new shoots (Maharjan *et al.*, 2019; Kolomiets *et al.*, 2022; Muharyati *et al.*, 2024). In the experiment, apical shoot segments measuring 0.6-1.2 mm in length were used. For morphogenesis induction, aseptic explants were transferred to MS media with varying compositions and concentrations of growth regulators (Table 6).

Table 6. Composition of culture media for organogenesis induction

No.	Medium component	Concentration in different medium variants (mL/L)				
		1	2	3	4	5
1	MS macronutrients	100	100	50	100	100
2	MS micronutrients	1	1	1	1	1
3	MS vitamins	1	1	1	1	1
4	Fe chelate	5	5	5	5	5
5	6-BAP	1.0	0.25	–	0.5	–
6	IAA	–	–	0.1	–	–
7	NAA	0.5	1	–	1	–
8	Myo-inositol (g/L)	0.1	0.1	0.1	0.1	0.1
9	Yeast extract (g/L)	0.5	0.5	–	1	–
10	Casein hydrolysate (g/L)	0.5	0.5	–	–	1
11	Sucrose (g/L)	–	30	30	–	30
12	Glucose (g/L)	20	–	–	15	–
13	Agar (g/L)	6.9	6.9	6.9	6.9	6.9

Source: developed by the authors

Explants were cultivated under a light intensity of 3-4 klx, with a 16-hour photoperiod, at 24°C ± 2°C and 70% relative humidity. The results were evaluated on the 30th day of *in vitro* cultivation. During this period, the growth of shoot length, the number of internodes, activation of meristems, and explant colour were monitored. An intermediate stage of microclonal propagation (MCP) involves producing a large number of microclones from a single initial explant and

promoting their intensive growth *in vitro*. The main factor in MCP is achieving a high multiplication rate, which is largely influenced by plant growth regulators and synthetic growth stimulators. For active growth of orchid cultures, auxins such as IAA and IBA, alongside 6-BAP, are recommended. In addition, essential components of the culture media include casein hydrolysate and yeast extract, which enhance explant development (Chugh *et al*., 2009). Between days 42 and 49 of cultivation, the formation of secondary shoots was observed. The number of shoots produced ranged from one to three per explant, with an average length of 2.6 cm (Fig. 5).

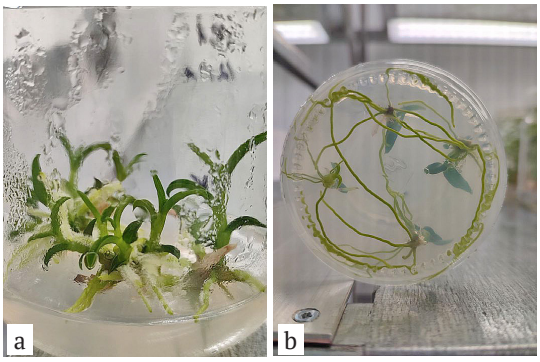

Figure 5. Regenerated plants from apical shoots of *Vanda* orchids
Note: a – 'Vanda coerulea'; b – 'Vanda sanderiana'
Source: provided by the authors

The results indicate that the optimal concentration of growth regulators, particularly 6-BAP and IAA, promotes the activation of apical meristems and the effective formation of new shoots, corroborating the findings of Y. Muharyati *et al*. (2024). This underscores the suitability of using apical shoots in microclonal propagation of orchids, as this approach enables the rapid production of a substantial quantity of high-quality, genetically uniform planting material. Studies of experimental morphogenesis *in vitro*, at various levels of organisation from single cells to complete organs, have provided the foundation for developing efficient microclonal propagation techniques (Kaeppler *et al*., 2000; Podhaetskyi *et al*., 2018; Kolomiets *et al*., 2022). A particularly promising approach involves the use of leaf explants containing remnants of aerial roots and callus to initiate shoot formation in *Vanda* species (Lang & Hang, 2006; Rahman *et al*., 2009). This method enhances the proliferation rate and accelerates the large-scale production of healthy planting material (Kaeppler *et al*., 2000; Podhaetskyi *et al*., 2018). The method of direct organogenesis involves the formation of new shoots directly from explant cells, bypassing the callus stage and thereby maintaining the genetic stability of the plant material. However, for certain *Vanda* orchid cultivars, the use of callus as an intermediate stage can enhance the morphogenetic potential of tissues, improving regeneration outcomes (Rahman *et al*., 2009; Nongdam *et al*., 2023).

Shoot formation in *Vanda* orchids occurs via two main mechanisms: the development of shoots from existing meristematic zones of the leaf near the base of the aerial root, or the formation of secondary meristems through the redifferentiation of specialised callus cells. Both mechanisms are influenced by growth regulators, particularly the cytokinin 6-BAP and the auxins IBA and NAA in appropriate ratios (Rahman *et al*., 2009; Hardjo *et al*., 2021). Leaf explants measuring 1.5-2 cm, including remnants of aerial roots, were used. Cultivation was carried out on modified MS medium supplemented with 1.5 mg/L 6-BAP and 0.2 mg/L IBA, under a temperature of 25°C ± 1°C, a 16hour photoperiod, light intensity of 3 klx, and relative humidity of 70-80%. By day 28, shoot formation was observed in 83% of explants, with the number of shoots per explant ranging from 1 to 5. By day 42, the average number of shoots per explant reached 66.7 ± 0.3, demonstrating the effectiveness of combined explants (leaf + aerial root) as a source of regeneration (Table 7 and Fig. 6).

Table 7. Shoot formation in 'Vanda coerulea' from combined explants (leaf + aerial root + callus)

Observation period	Shoots 1-2.9 cm	Shoots 3-4.9 cm	Shoots ≥5 cm	Total shoots
'Vanda coerulea'				
28 days	42 ± 2.1	18 ± 0.9	6 ± 0.3	66 ± 3.3
42 days	58 ± 2.9	32 ± 1.6	12 ± 0.6	102 ± 5.1

Source: developed by the authors

Figure 6. Regenerated 'Vanda sanderiana' plants from leaf explants with aerial roots and callus
Source: provided by the authors

The shoot formation process was accompanied by active proliferation of callus tissue at the base of the explants. Subsequent differentiation of these structures into apical organs indicates a high morphogenetic potential of these organogenic centres (Hardjo *et al.*, 2021; Nongdam *et al.*, 2023). Thus, the results confirm the efficacy of using leaf explants with remnants of aerial roots and callus for regeneration in *Vanda* orchids. This approach achieves high micropropagation efficiency, minimises the risk of somaclonal variation, and allows the mass production of virus-free planting material for industrial applications. The regenerated plants serve as material for further cloning, thereby increasing the multiplication coefficient.

Induction of morphogenesis in the introduced *Vanda* explants

For morphogenesis induction, sterile explants were transferred to fresh nutrient media containing varying concentrations of growth regulators and 2 g/L activated charcoal. Explants were cultivated under a light intensity of 3-4 klx, with a 16-hour photoperiod, at 24°C ± 2°C and 70% relative humidity. Results were assessed on day 30 of *in vitro* cultivation. During the cultivation period, the formation of shoot length, the number of internodes, the activity of meristems, and the colour of the explants were monitored. An intermediate stage in micropropagation involves generating a large number of microclones from a single initial explant and promoting their vigorous growth *in vitro*. Cultivation of orchids on media No. 3 and No. 5 produced negative outcomes due to tissue oxidation caused by phenolic compounds (Fig. 7). Media No. 1 and No. 2 are recommended for active growth, development, and propagation, as they support rapid protocorm enlargement and the formation of new leaves and roots by day 15 of cultivation (Fig. 8).

Figure 7. Phenolic auto-toxicity in a 'Vanda coerulea' explant on medium No. 3 on day 10 of cultivation
Source: provided by the authors

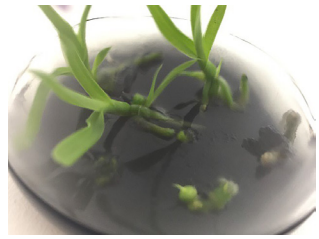

Figure 8. 'Vanda coerulea' on medium No. 2 on day 15 of cultivation
Source: provided by the authors

Thus, morphogenesis in both Vanda orchid cultivars is most intensive on media supplemented with 6-BAP (1.0 mg/L), NAA (0.5 mg/L), yeast extract (0.5 g/L), casein hydrolysate (0.5 g/L), and activated charcoal (2 mg/L). Under these conditions, explants reached 2.5-4.0 cm in size, acquired a deep green colour, and exhibited activation of dormant buds and protocorm growth. This combination of cytokinins and auxins is considered optimal for overcoming apical dominance in the *Orchidaceae* family. This aligns with the findings of L. Tikendra *et al*. (2025), who reported the most effective morphogenesis on MS medium supplemented with 1.2 mg/L kinetin and 0.6 mg/L IAA. On medium containing IAA (0.1 mg/L) without activated charcoal, phenolic exudation was pronounced, explants appeared weak, and dormant bud activation did not occur. When 6-BAP (0.5 mg/L) and NAA (1.0 mg/L) were added to the medium, explants

remained small (0.5-1.0 cm), pale in colour, and exhibited only weak bud sprouting.

Rooting of regenerated *Vanda* orchids *in vitro*

Root formation in regenerated plants represents the final and simultaneously one of the most challenging stages of micropropagation for orchids, particularly for *Vanda* species. Successful development of a functional root system is critical for subsequent growth under *in vivo* conditions (Thomas, 2008; Kumar & Rao, 2012; Maharjan *et al*., 2019). A distinctive feature of *Vanda* orchids is the production of aerial roots, which are covered with velamen – a specialised tissue that absorbs moisture from the air and participates in photosynthesis. Therefore, successful rhizogenesis requires not only the correct medium but also adequate access to oxygen and light. Roots develop most effectively under diffuse natural light or cool artificial lighting at an intensity of 2-3 klx.

In the studies of T. Murashige & F. Skoog (1962) and G.P. Kushnir & V.V. Sarnatska (2005), the use of half-strength MS medium without added growth regulators was recommended to stimulate root system development. However, A. Setiaji *et al*. (2021) and Y. Muharyati *et al*. (2024) demonstrated the advantages of including auxins, such as NAA or IBA. The effect of different NAA concentrations (0.5-2.0 mg/L) on root formation in microshoots of various *Vanda* orchid cultivars was examined using agarised MS medium with half-strength mineral salts. Standard-sized shoots with well-developed leaf rosettes were selected for the experiment and placed on several nutrient medium variants. The results are summarised in Table 8. The highest rooting percentage (87.5%) was observed on the MSR2 medium supplemented with 0.5 mg/L NAA (Fig. 9). By contrast, MSR1 and MSR3 showed considerably lower rooting rates of 30% and 55%, respectively.

Table 8. Effect of nutrient medium composition on the rooting of *Vanda* 'Vanda coerulea' shoots

Variant	Medium composition	Number of shoots (pcs)	Number of rooted shoots (pcs)	Rooting, %
MSR1	½ MS	40	12 ± 0.6	30 ± 1.5
MSR2	½ MS + 0.5 mg/L NAA	40	35 ± 1.8	87.5 ± 4.4
MSR3	½ MS + 2.0 mg/L NAA	40	22 ± 1.4	55 ± 2.8

Source: developed by the authors

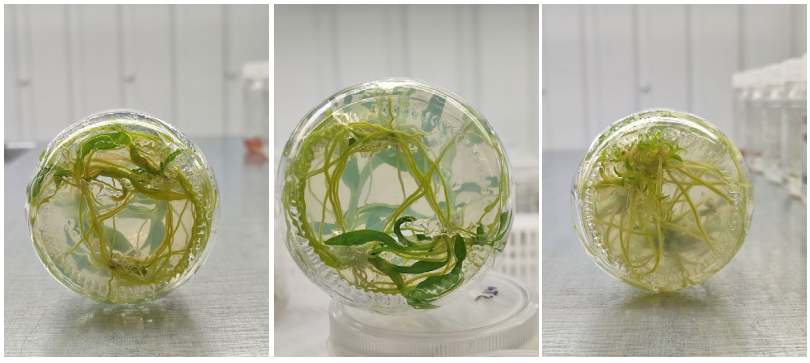

Figure 9. Rooted regenerated 'Vanda coerulea' plants on MSR2 medium
Source: provided by the authors

Based on the results of the study, the most effective medium for stimulating root system development in regenerated *Vanda* orchids was MS medium with half-strength macro- and microelements, supplemented with 0.5 mg/L NAA. This medium promoted a high rooting rate, which is critical for successful root system development and the subsequent adaptation of plants to *in vivo* conditions. Therefore, the use of this medium is recommended for integration into *Vanda* orchid micropropagation protocols, ensuring consistent and reliable outcomes.

Acclimatisation of regenerated *Vanda* orchids to *in vivo* conditions

Plants with well-developed root systems and healthy, dense green leaves were removed from the nutrient medium for subsequent acclimatisation. To facilitate adaptation to a non-sterile environment, the culture vessels were opened, and 2-3 mL of sterile distilled water was added, leaving the plants under these conditions for two days. The plants were then carefully removed, washed under running water to remove any residual agar medium from the roots, and disinfected with a 1% potassium permanganate solution to prevent potential infections. Prepared plants were planted in a substrate composed of sphagnum moss, peat, shredded beech leaves, and pine bark in a 1:1:1:1 ratio. Occasionally, additional components such as charcoal, fallen leaves, or dolomite flour are added to improve substrate properties; however, the core substrate typically consists of various proportions of bark, moss, peat, and fern roots. The pots with planted orchids were placed under conditions of high humidity to promote successful establishment. After 10-12 days, as the plants adapted, they established successfully and began to grow. The survival rates of regenerated plants of different *Vanda* orchid cultivars are presented in Table 9.

Table 9. Survival of regenerated plants of different *Vanda* orchid cultivars

Cultivar	Number of plants planted in substrate (pcs)	Number of plants established	
		pcs	%
'Vanda coerulea'	40	35	87.5 ± 4.4
'Vanda sanderiana'	40	33	82.5 ± 4.1

Source: developed by the authors

By the 30th day of cultivation, the seedlings had reached a height of 8-9 cm and developed 6-8 true leaves, making them suitable for transplanting into open-ground greenhouse conditions (Fig. 10). The substrate for *Vanda* orchids should be loose, moisture-retentive, and nutrient-rich, although nutrient content is less critical and can be adjusted through fertilisation. Most *Vanda* orchids do not have a clearly defined dormancy period, so watering should be moderate but regular throughout the year. The substrate should remain moist but not waterlogged, and water at room temperature should be used. Various types of containers can be used for planting, including ceramic or plastic pots, baskets, or mesh holders.

Figure 10. Regenerated 'Vanda sanderiana' adapted to *in vivo* conditions
Source: provided by the authors

Thus, the adaptation study of regenerated plants of different *Vanda* cultivars demonstrated that the survival rate of 'Vanda coerulea' under *in vivo* acclimatisation conditions was 87.5%, while under the same conditions, 'Vanda sanderiana' achieved 83%. These results indicate a high survival capacity for both cultivars, with 'Vanda coerule'a showing slightly better performance.

CONCLUSIONS

The conducted research yielded aseptic, viable explants of cultivars 'Vanda coerulea' and 'Vanda sanderiana', which were subsequently used for studies on callus induction, morphogenesis, and plant regeneration. It was determined that the most effective callus-inducing medium for both cultivars of *Vanda* orchids was MS medium supplemented with 2.5 mg/L NAA and 0.4 mg/L 6BAP. Under these conditions, 99-100% of explants formed callus, with a fresh callus mass of 690 mg for 'Vanda coerulea' and 655 mg for 'Vanda sanderiana'. The efficiency of indirect morphogenesis in *Vanda* orchids depends on several factors, including the genotype of the donor plant, the presence of growth regulators in the culture medium, and the number of passages of callus tissue. To maintain high morphogenetic activity, it is recommended to limit the number of passages to 5-6 and

to periodically renew the initial callus. The results also confirm the suitability of using leaf explants with remnants of aerial roots and callus for regeneration in *Vanda* orchids. This approach allows for highly efficient micropropagation, minimises the risk of somaclonal variation, and enables the mass production of virus-free planting material for commercial purposes. The regenerated plants provide material for further cloning, thereby increasing the propagation coefficient. Morphogenesis in both Vanda cultivars was most vigorous on a medium supplemented with 6-BAP (1.0 mg/L), NAA (0.5 mg/L), yeast extract (0.5 g/L), casein hydrolysate (0.5 g/L), and activated charcoal (2 mg/L). The most effective medium for stimulating root system formation in regenerated *Vanda* plants was MS medium with half-strength macro- and microelements, supplemented with 0.5 mg/L NAA. This formulation ensures a high level of rooting, making it suitable for inclusion in micropropagation protocols for this genus. During the acclimation of regenerated plants, survival rates for the cultivar 'Vanda coerulea' reached 87.5%, while under identical conditions, 'Vanda sanderiana' achieved 83%. These results provide a basis for developing standardised propagation protocols for *Vanda* orchids, ensuring a high level of control at all stages of cultivation. Implementation of such protocols is critical for breeding programmes, the conservation of rare cultivars, and the improvement of plant material quality for commercial horticulture.

ACKNOWLEDGEMENTS

None.

FUNDING

None.

CONFLICT OF INTEREST

None.

REFERENCES

[1] Baby, R., Valsala, P.A., & Manjesh, S. (2019a). Genetic fidelity evaluation of micropropagated plantlets of *Vanda hybrid* Dr. Anek. *International Journal of Current Microbiology and Applied Sciences*, 8(8), 2373-2381. doi: 10.20546/ijcmas.2019.808.276.

[2] Baby, R., Valsala, P.A., & Doddamani, M.B. (2019b). *In vitro* micropropagation protocol for *Vanda hybrid* 'dr. Anek.' *International Journal of Current Microbiology and Applied Sciences*, 8(4), 2073-2084. doi: 10.20546/ijcmas.2019.804.244.

[3] Bhattacharjee, B., & Islam, S.M.S. (2014a). Development of an efficient protocol for *in vitro* germination and enhancing protocorm-like body development in three indigenous orchid species in Bangladesh. *Asian Pacific Journal of Molecular Biology and Biotechnology*, 22(3), 209-218.

[4] Bhattacharjee, B., & Islam, S.M.S. (2014b). Effects of plant growth regulators on multiple shoot induction in *Vanda tessellate* (Roxb.) Hook. Ex G.Don an endangered medicinal plant. *International Journal of Science and Nature*, 5, 707-712.

[5] Chen, J.-T. (2018). *In vitro* organogenesis of a slipper orchid, paphiopedilum 'Alma Gavaert'. *Notulae Scientia Biologicae*, 10(4), 607-613. doi: 10.15835/nsb10410368.

[6] Chugh, S., Guha, S., & Rao, I.U. (2009). Micropropagation of orchids: A review on the potential of different explants. *Scientia Horticulturae*, 122(4), 507-520. doi: 10.1016/j.scienta.2009.07.016.

[7] Convention on Biological Diversity. (1992, June). Retrieved from https://surl.lu/mrezfd.

[8] Dar, S.A., Nawchoo, I.A., Tyub, S., & Kamili, A.N. (2021) Effect of plant growth regulators on *in vitro* induction and maintenance of callus from leaf and root explants of *Atropa acuminata* Royal ex. Lindl. *Biotechnology Reports*, 32, article number e00688. doi: 10.1016/j.btre.2021.e00688.

[9] Davis, B.J., Phillips, R.D., Wright, M., Linde, C.C., & Dixon, K.W. (2015). Continent-wide distribution in mycorrhizal fungi: Implications for the biogeography of specialized orchids. *Annals of Botany*, 116(3), 413-421. doi: 10.1093/aob/mcv084.

[10] Hardjo, P.H., Savitri, W.D., Artadana, I.B.M., Putra, S.E.D., Parac, E.P., & Jan, A. (2021). Callus-mediated somatic embryogenesis and plant regeneration in *Vanda tricolor* Lindl. var. Pallida. *Jordan Journal of Biological Sciences*, 14(05), 933-937. doi: 10.54319/jjbs/140508.

[11] Kaeppler, S.M., Kaeppler, H.F., & Rhee, Y. (2000). Epigenetic aspects of somaclonal variation in plants. *Plant Molecular Biology*, 43, 179-188. doi: 10.1023/a:1006423110134.

[12] Kolomiets, Yu.V., Kliachenko, O.L., & Subin, O.V. (2022). *Biotechnology*. Kyiv: Comprint.
[13] Kumar, K., & Rao, I.U. (2012). Morphophysiologicals problems in acclimatization of micropropagated plants in -*ex vitro* conditions- a reviews. *Journal of Ornamental Plants*, 2, 271-283.
[14] Kushnir, G.P., & Sarnatska, V.V. (2005). *Microclonal propagation of plants*. Kyiv: Naukova Dumka.
[15] Laily, A.N., Maharijaya, A., Wulandari, D., Puspitaningtyas, D.M., Ariate, S.R., Martin, A.F., & Hapsari, B.W. (2024). Optimizing micropropagation of Indonesian conserved orchid *Vanda celebica* using organic compunds and a temporary immersion system. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 52(4), article number 14065. doi: 10.15835/nbha52414065.
[16] Lang, N.T., & Hang, N.T. (2006). Using biotechnological approaches for *Vanda* orchid improvement. *Omonrice*, 14, 140-143.
[17] Lee, Y.-I., Hsu, S.-T., & Yeung, E.C. (2013). Orchid protocorm-like bodies are somatic embryos. *American Journal of Botany*, 100(11), 2121-2131. doi: 10.3732/ajb.1300193.
[18] Likhanov, A.F., Overchenko, O.V., Kostenko, S.M., & Subin, O.V. (2017). Specificity of cytodifferentiation in calluses *in vitro* of *Aesculus Hippocastanum* form resistant to horse-chestnut leaf miner. *Plant Physiology and Genetics*, 49(6), 495-505. doi: 10.15407/frg2017.06.495.
[19] Liu, X.-f., Xiang, L.-l., Huang, Y., Li, Y.-j., & Li, F. (2020). Efficient propagation with *in vitro* seed germination of *Vanda falcata*. *Journal of Zhejiang University-SCIENCE B*, 21, 999-1004. doi: 10.1631/jzus.b2000505.
[20] Maharjan, S., Pradhan, S., Thapa, B.B., & Pant, B. (2019). *In vitro* propagation of endangered orchid, *Vanda pumila* Hook.f. through protocorms culture. *American Journal of Plant Sciences*, 10(07), 1220-1232. doi: 10.4236/ajps.2019.107087.
[21] Muharyati, Y., Setiyadi, M.W., Khatimah, A., & Ardiansyah, A. (2024). Propagation of the *Vanda helvola* orchid *in vitro*. *Jurnal Pijar Mipa*, 19(3), 488-492. doi: 10.29303/jpm.v19i3.6600.
[22] Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15, 473-497. doi: 10.1111/j.1399-3054.1962.tb08052.x.
[23] Nongdam, P., Beleski, D.G., Tikendra, L., Dey, A., Varte, V., EL Merzougui, S., Pereira, V.M., Barros, P.R., & Vendrame, W.A. (2023). Orchid micropropagation using conventional semi-solid and temporary immersion systems: A review. *Plants*, 12(5), article number 1136. doi: 10.3390/plants12051136.
[24] Nowakowska, K., Marciniak, P., & Pacholczak, A. (2022). A protocol for efficient micropropagation of rare orchid *Vanda brunnea* Rchb.f. *South African Journal of Botany*, 150, 233-239. doi: 10.1016/j.sajb.2022.07.023.
[25] Palama, T.L., Menard, P., Fock, I., Choi, Y.H., Bourdon, E., Govinden-Soulange, J., Bahut, M., Payet, B., Verpoorte, R., & Kodja, H. (2010). Shoot differentiation from protocorm callus cultures of *Vanilla planifolia* (Orchidaceae): Proteomic and metabolic responses at early stage. *BMC Plant Biology*, 10(1), article number 82. doi: 10.1186/1471-2229-10-82.
[26] Pathak, P., Kumari, A., Chandler, B.D., & Zettler, L.W. (2023). *In vitro* propagation and phytochemical analysis of *Vanda cristata* Wall. ex Lindl: An endangered medicinal orchid of biopharmaceutical importance. *South African Journal of Botany*, 153, 109-123. doi: 10.1016/j.sajb.2022.11.023.
[27] Podhaetskyi, A.A., Matskevych, V.V., & Podhaetskyi, A.A. (2018). *Features of microclonal propagation plant species: Monography*. Bila Tserkva: BNAU.
[28] Rahman, M., Hasan, M., Das, R., Hossain, M., & Rahman, M. (2009). *In vitro* micropropagation of orchid (*Vanda tessellata* L.) from shoot tip explant. *Journal of Bio-Science*, 17, 139-144. doi: 10.3329/jbs.v17i0.7122.
[29] Rasmussen, H.N., & Rasmussen, F.N. (2014). Seedling mycorrhiza: A discussion of origin and evolution in *Orchidaceae*. *Botanical Journal of the Linnean Society*, 175(3), 313-327. doi: 10.1111/boj.12170.
[30] Setiaji, A., Annisa, R.R., Santoso, A.D., Kinasih, A., & Riyadi, A.D. (2021). Review: Factors affecting mass propagation of *Vanda orchid in vitro*. *Cell Biology and Development*, 5(2). doi: 10.13057/cellbioldev/v050201.

[31] Shemediuk, N., & Skip, O. (2014) Application biotechnological methods for the biodiversity representative of the family *Orchidaceae Juss*. *Scientific Bulletin of the S.Z. Gzhytskyi National University of Veterinary Medicine and Biotechnologies Lviv*, 16(3), 345-357.
[32] Sheyko, O.A., & Musatenko, L.I. (2013). The induction callus formation *in vitro* from explants of rare and endangered orchid. *Chornomorski Botanical Journal*, 9(4), 595-604.
[33] Thomas, T.D. (2008). The role of activated charcoal in plant tissue culture. *Biotechnology Advances*, 26(6), 618-631. doi: 10.1016/j.biotechadv.2008.08.003.
[34] Tikendra, L., Singh, A.R., Vendrame, W.A., & Nongdam, P. (2025). *In vitro* propagation of endangered 'Vanda coerulea' Griff. ex Lindl.: Asymbiotic seed germination, genetic homogeneity assessment, and micro-morpho-anatomical analysis for effective conservation. *Agronomy*, 15(5), article number 1195. doi: 10.3390/agronomy15051195.

Мікроклональне розмноження орхідеї *Vanda* (*Orchidaceae Vanda*)

Оксана Кляченко

Доктор сільськогосподарських наук, професор
Національний університет біоресурсів і природокористування України
03041, Героїв Оборони, 15, м. Київ, Україна
https://orcid.org/0000-0002-4087-4082

Олександр Субін

Кандидат біологічних наук, доцент
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
https://orcid.org/0000-0001-6209-2439

Ольга Олійник

Кандидат сільськогосподарських наук
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
https://orcid.org/0000-0002-3591-3518

Діана Шишкіна

Студент
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
https://orcid.org/0009-0001-2486-7691

Анотація. Біотехнологічні методи розмноження орхідеї *Vanda* є ефективним інструментом отримання великої кількості генетичнооднорідного оздоровленого рослинного матеріалу. Метою роботи було вивчення особливостей мікроклонального розмноження орхідеї *Vanda*. Для отримання посадкового матеріалу різних генотипів орхідеї *Vanda*, а саме: 'Vanda coerulea' та 'Vanda sanderiana' методом мікроклонального розмноження використано прямий, непрямий морфогенез і ризогенез *in vitro* та адаптація до умов *in vivo*. Розроблено ступінчатий метод отримання асептичного матеріалу, який полягає у використанні 70 % етилового спирту протягом 1 хвилини з подальшим використанням основного стерилянту 2,5 % NaClO протягом 5-10 хв, що уможливлює отримання 70-80 % стерильних життєздатних експлантатів, знижуючи рівень контамінації грибами та бактеріями. Представлено результати прямого та непрямого морфогенезу, калюсогенезу і ризогенезу в культурі *in vitro* експлантатів орхідеї *Vanda*. Встановлено, що між сортами орхідеї не спостерігалося значних відмінностей у процесах калюсогенезу. Частота калюсогенезу для обох генотипів становила 100 %, що вказує на високу здатність до утворення калюсної тканини. Найкращі результати росту та пагоноутворення були досягнуті на живильному середовищі Мурасіге-Скуга (МС), доповнене 6-Бензиламінопурином (6-БАП) у концентрації

0,5 мг/л. Для укорінення найбільш ефективним виявилося середовище МС з половинною концентрацією макро- та мікросолей, доповнене 0,5 мг/л α-нафтилоцтовою кислотою (НОК), яке рекомендоване для ризогенезу рослин-регенерантів орхідеї *Vanda* різних сортів. Для адаптації рослин-регенерантів використано субстрат з сфангового моху, торфу, подрібнених листків бука та соснової кори у співвідношенні 1:1:1:1. Приживаність рослин орхідеї до умов *in vivo* сорту 'Vanda coerulea' становила 87,5 %, тоді як за однакових умов у рослин сорту 'Vanda sanderiana' – 83 % відповідно. Отримані результати досліджень можуть бути основою для створення протоколів розмноження орхідеї *Vanda*, що забезпечить високий рівень контролю на всіх етапах вирощування. Впровадження таких протоколів є важливими для селекційної роботи, збереження рідкісних сортів та поліпшення якості рослинного матеріалу для промислового квітникарства

Ключові слова: *in vitro*; калюсогенез; морфогенез; різогенез; адаптація *in vivo*

Received: 07.07.2025 Revised: 30.10.2025 Accepted: 27.11.2025 Published: 26.12.2025

BIOLOGICAL SYSTEMS: Theory and Innovation

ISSN 2706-8382
e-ISSN 2706-8390

Journal homepage: https://bioscience.com.ua/en
Vol. 16, No. 4, 2025, 57-70

UDC 632.38:634.11:577.21

DOI: 10.31548/biologiya/4.2025.57

Molecular identification and monitoring of major apple tree viruses in Ukraine

Kateryna Hrynchuk

PhD in Biological Sciences, Associate Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
https://orcid.org/0000-0002-3138-0080

Ihor Antipov*

PhD in Agricultural Sciences, Associate Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
https://orcid.org/0000-0002-1156-267X

Abstract. Viral diseases of apple trees cause significant economic losses in horticulture in Ukraine, which requires an updated research into their prevalence. The purpose of the study was to assess the prevalence and regional distribution of the main apple tree viruses in industrial plantings of Ukraine using molecular diagnostic methods. The study was conducted by reverse transcription and polymerase chain reaction on 350 samples of apple leaves and bark taken from industrial plantings in five regions of Ukraine. The specificity of detection of three target viruses was confirmed by sequencing the obtained amplicons. The study found that the overall infection rate of apple plantations was 70.6%, which indicates an epiphytic situation. The most prevalent was *Apple stem pitting virus* (46.0%), whereas *Apple chlorotic leaf spot virus* and *Apple mosaic virus* were detected in 36.0% and 13.7% of samples, respectively. Regional analysis revealed the maximum prevalence of viruses in the Zakarpattia (86.0%) and Kyiv (76.3%) regions, while the minimum indicators were recorded in the Kharkiv region (56.8%). A high level of mixed infections was found (25.4%), with a predominance of the combination of *Apple stem pitting virus* and *Apple chlorotic leaf spot virus* (18.3%). Also significant is the high percentage of asymptomatic infections (31.2%), which complicates phytosanitary control. The conclusions confirmed that the results obtained justify the need for urgent implementation of a system for molecular monitoring and certification of planting material. The results of the study can be used to develop a national programme for improving apple plantations in Ukraine

Keywords: polymerase chain reaction; apple stem pitting virus; apple chlorotic leaf spot virus; apple mosaic virus

INTRODUCTION

Apple orchards occupy a leading place in horticulture in Ukraine, but their productivity is significantly limited by viral diseases. The global problem of the spread of viruses on fruit crops is becoming particularly relevant in the context of the growth of intensive gardening. Studies conducted in different regions of the world show trends in the high level of infection of apple plantations.

Suggested Citation:

Hrynchuk, K., & Antipov, I. (2025). Molecular identification and monitoring of major apple tree viruses in Ukraine. *Biological Systems: Theory and Innovation*, 16(4), 57-70. doi: 10.31548/biologiya/4.2025.57.

*Corresponding author

In particular, L. Ma *et al.* (2021) found a high prevalence of major pear viruses in commercial orchards in China, indicating the severity of the problem in the East Asian region. The study included analysis of samples from various provinces of China, where the presence of pathogens such as *Apple stem pitting virus* and *Apple chlorotic leaf spot virus*. The researchers applied advanced molecular diagnostic methods, which helped to obtain representative data on the epidemiology of viral diseases in fruit crops. Similar studies in Kazakhstan conducted by N. Kerimbek *et al.* (2024) showed a high level of viral damage to apple plantations, which requires the development of effective control measures. The researchers conducted monitoring in the southern regions of Kazakhstan using serological and molecular diagnostic methods. The study revealed significant variability in the spread of different viruses depending on the variety and age of the plantings.

Advanced diagnostics of plant viruses is based on molecular methods that allow accurate identification of pathogens. S. Heo & Y.S. Chung (2020) developed advanced PCR (polymerase chain reaction) techniques for detecting apple viruses, which significantly improved diagnostic accuracy. The researchers optimised PCR conditions for sensitive and specific detection of *Apple stem grooving virus* and *Apple chlorotic leaf spot virus*. The developed methods have demonstrated high efficiency in analysing samples with different types of infections. A notable achievement is the development by D.A. Gritsenko *et al.* (2020) of multiplex PCR method for simultaneous detection of five apple viruses, which simplifies the mass screening process. The method allows saving time and reagents when conducting large-scale research. The researchers tested the specificity and sensitivity of the method on a wide collection of samples. In Latvia, N. Zuļģe *et al.* (2023) conducted an in-depth phylogenetic study of ACLSV isolates, revealing significant genetic diversity of the pathogen. The study included analysis of isolates from different host species – *Malus, Pyrus* and *Prunus*. Phylogenetic analysis revealed a clear clustering of isolates by host species and geographical origin.

In Ukraine, the problems of viral diseases of agricultural crops were studied mainly on vegetable crops, which was confirmed by V.O. Tsvigun *et al.* (2024). The researchers monitored viral infections in vegetable agrocoenoses using advanced molecular diagnostic methods. The study covered the analysis of samples from different regions of Ukraine, which helped to identify the species composition of viral pathogens. The results obtained indicate the need to introduce a system for regular monitoring of viral diseases of agricultural crops. Research by L.T. Mishchenko *et al.* (2022) on the Wheat dwarf virus demonstrated the relevance of the problem of viral diseases in agrocoenoses of Ukraine. The researchers conducted molecular characterisation of local isolates of the virus, investigating their genetic diversity. A link was established between the spread of the pathogen and grain yield. However, comprehensive studies of apple tree viruses remain rather neglected, which creates a significant gap in the development of an effective plant protection system. Lack of up-to-date data on the prevalence of pathogens such as *Apple stem pitting virus, Apple chlorotic leaf spot virus,* and *Apple mosaic virus* complicates the development of targeted phytosanitary measures. The existing information is based mainly on outdated diagnostic methods that do not meet modern requirements.

International experience shows the effectiveness of advanced methods of combating viral infections. Research by Korean scientists Y.H. Kwon *et al.* (2024) showed high efficiency of a combination of thermotherapy and chemicals for improving the health of planting material. The researchers achieved a significant reduction in the concentration of viruses in plant material by optimising temperature conditions. In Kazakhstan, G. Kairova *et al.* (2023) successfully applied molecular markers to identify varieties resistant to bacterial infections, opening up prospects for similar studies of viral diseases. The findings showed the possibility of rapid identification of resistance genes using PCR analysis. Given the global nature of the problem of the spread of apple viruses and the lack of data on the situation in Ukraine, there is a need for a comprehensive study of the prevalence of the main viral pathogens in apple plantations in different regions of the country. Existing studies, such as research by L. Pavliuk *et al.* (2021) on cherry viruses, confirmed the relevance of such studies for fruit crops in Ukraine, but there are no specialised studies on apple trees. The researchers used molecular methods to detect the main

viruses of stone crops, which allowed assessing the phytosanitary condition of plantings.

The purpose of the study was to carry out a comprehensive assessment of the phytosanitary condition of industrial apple plantations in Ukraine by investigating the prevalence of major viral pathogens (ASPV (*Apple stem pitting virus*), ACLSV (*Apple chlorotic leaf spot virus*), and AMV (*Apple mosaic virus*)), for which the following tasks were set: to conduct molecular monitoring using the method of reverse transcription and polymerase chain reaction to identify the main viral pathogens; to analyse the regional features of the distribution of viral infections in the main areas of industrial apple cultivation; to assess the level of mixed infections and determine the proportion of latent forms of damage.

MATERIALS AND METHODS

The process of selecting plant material was organised to ensure the representativeness of samples from the main areas of industrial apple cultivation in Ukraine. The sampling was carried out during two growing seasons – from May to August 2023 and 2024. 350 samples of leaves and young bark of apple trees of the 'Golden Delicious' (100 samples), 'Idared' (90 samples), 'Gala' (80 samples), and 'Champion' (80 samples) varieties were selected. The sampling was carried out in industrial gardens of five regions of Ukraine characterised by different climatic conditions: Kyiv (n = 80), Vinnytsia (n = 80), Cherkasy (n = 70), Kharkiv (n = 70), and Zakarpattia (n = 50). Leaf samples were taken from the middle tier of the crown from different cardinal directions, and bark samples were taken from annual increments. Attention was paid to apple trees with pronounced symptoms of viral damage. In particular, samples were taken with the following signs: leaf mosaic (characteristic light green or yellow spots and stripes) – a classic symptom of infection with the *Apple mosaic virus* (ApMV). Chlorosis (yellowing along the veins or inter-vein chlorosis) was caused by *Apple chlorotic leaf spot virus* (ACLSV). Deformation of the leaf plate (twisting, asymmetry, threadiness) is associated with infection with the *Apple stem pitting virus* (ASPV) or a complex of the mentioned viruses. However, to assess the latent spread of infection, some of the material was also taken from clinically healthy plants, since many viruses (for example, ACLSV and ASPV) circulate in gardens without visible symptoms, forming so-called latent complexes of apple viruses. All samples were labelled, indicating the date, place of selection, grade, and description of the phytopathological condition, and then transported in ice containers manufactured by Thermo Fisher Scientific (USA) to the laboratory, where they were stored at -80°C until further analysis. All further stages of RNA (ribonucleic acid) and molecular diagnostics were performed under controlled laboratory conditions.

Total RNA isolation was performed from 100 mg of plant tissue, which was previously ground in liquid nitrogen with a mortar. Nucleic acid extraction was performed using the commercial kit "Ukrbiopreparat" (Ukraine), based on the method of heterophase extraction with silicon dioxide and detergent (Bogner & Killeen, 2006). Specifically, 500 µL of lysing buffer was added to the crushed tissue, the mixture was incubated at 65°C for 10 minutes, then 250 µL of chloroform was added and thoroughly mixed on vortex. After cenrifugation at 12,000 RCF for 10 minutes, the aqueous phase was carefully transferred to a new microcentrifuge tube, where RNA deposition was performed by adding an equal volume of isopropanol and incubation at -20°C for 30 minutes. After repeated centrifugation, the resulting RNA was washed in 70% ethanol, air-dried, and dissolved in 30 µL of RNA-free water. The quality and quantity of the extracted RNA were assessed using two methods: spectrophotometrically with a NanoDrop system (Deben *et al.*, 2013), where samples with an A260/280 ratio in the range of 1.8-2.1 were considered acceptable; and electrophoretically by fractionation of 2 µL of RNA in a 1% agarose gel stained with ethidium bromide (Barros *et al.*, 2018), where RNA integrity was confirmed by the presence of distinct 18S and 28S rRNA bands.

Reverse transcription was performed at a volume of 20 µL using 1 µg of total RNA, 100 Ng of random hexamer primers, 20 units of M-MuLV revertase, and a buffer containing 10 mM DTT, 1 mm dNTP mixture, and 20 units of an RNase inhibitor. The reaction was performed according to the following temperature programme: 25°C for 5 minutes for primer hybridisation, 42°C for 60 minutes for cDNA synthesis, and 70°C for 5 minutes for enzyme inactivation. The resulting cDNA was

diluted in a ratio of 1:5 with sterile water and stored at -20°C. The polymerase chain reaction was tuned to a volume of 25 µL, the reaction mixture included 2.5 µL of diluted cDNA, 1 x Taq polymerase buffer, 1.5 mm $MgCl_2$, 0.2 mm dNTP, 0.4 µM of each of the specific primers, and 1 unit of Taq DNA polymerase.

Molecular diagnostics of apple tree samples was performed by reverse transcription and polymerase chain reaction (RT-PCR). Published pairs of primers specific to conserved regions of their genome were used to detect the main apple viruses. For detection of ASPV, primers ASPV-F (5′-GACTGCATCTGGTTCAAC-3′) and ASPV-R (5′-CGCTTGATTTGGACTGTC-3′) were used, which amplify a 327 bp fragment of the coat protein (CP) gene (Ji *et al*., 2013; Hao *et al*., 2016). For detection of ACLSV, primers ACLSV-F (5′-GCGATGAAGTTGCTGATGA-3′) and ACLSV-R (5′-TGACCTCCATTGCCAAT-3′) were used, targeting a 458 bp region of the replicase gene (ORF1) (Ji *et al*., 2013). For analysis of ApMV, primers ApMV-F (5′-CAGAGCAACGCAGACTCA-3′) and ApMV-R (5′-GAGCCATACCAAACCAAT-3′) were used to amplify a 392 bp fragment of the coat protein (CP) gene (Ji *et al*., 2013).

Amplification was performed in the Applied Biosystems Veriti thermal cycler (USA) according to the standard protocol, which included initial denaturation at 94°C for 5 minutes, then 35 cycles, each of which consisted of denaturation at 94°C – 30 seconds, annealing of primers at 55°C – 30 seconds and elongation at 72°C – 45 seconds, followed by final elongation at 72°C for 7 minutes. PCR products were visualised by electrophoresis in 1.5% agarose gel using the Horizon horizontal electrophoresis system (Thermo Fisher Scientific, USA) and a power source (Bio-Rad, USA). The gel provided sufficient resolution to differentiate the expected amplicon sizes. The gel was stained with ethidium bromide, and the strips were visualised in UV light (UVP transilluminator BioDoc-It, USA) using a gel documentation system (Bio-Rad, USA). Samples with a clear band of the expected size corresponding to the positive control were considered positive. To confirm the specificity of amplification, random samples from each group of positive samples were selected for sequencing using the Sanger method (Crossley *et al*., 2020) on the Applied Biosystems 3500 analyser (USA). To quantify the relationship between clinical manifestations and the results of molecular detection of viruses, a standardised symptom intensity scale was developed in the range from 0 to 5 points, where 0 indicated the absence of symptoms, and 5 – the maximum severity of pathological signs. Visual evaluation was performed independently by three researchers, followed by averaging the results, and for each sample, the type of symptoms (mosaic, chlorosis, leaf deformity, necrosis) and their severity were recorded.

Statistical processing of the obtained data was performed using the Statistica 10.0 software suite (USA). The chi-square (χ^2) criterion was used to assess the relationship between the selection region, apple variety, and virus infection rate. The differences were considered statistically significant at the significance level $p < 0.05$. The prevalence of each virus was calculated as the percentage of positive samples from the total number tested. Pearson correlation analysis with calculation of the correlation coefficient (r) for quantitative data and analysis of conjugacy tables using the χ^2 criterion for categorical variables were used to identify patterns in the spread of mixed infections. All calculations were performed using the Statistica 10.0 software suite. All laboratory experiments, including RNA isolation and PCR analysis, were performed in three biological and two analytical repetitions to minimise random errors and ensure reproducibility of the results. During field research, the principles of biodiversity conservation were observed, in accordance with the provisions of the Convention on Biological Diversity (2025), and sampling was carried out with minimal impact on plants and their environment.

RESULTS AND DISCUSSION

General prevalence of viral infections in apple plantations

A molecular study using reverse transcription and polymerase chain reaction (RT-PCR) revealed a high level of infection of apple plantations with major viruses (ASPV, ACLSV, and ApMV) in Ukraine. Analysis of 350 samples of leaves and bark showed that 247 samples had a positive reaction to the presence of at least one of the three viruses under study. This indicator is 70.6% of the total sample size, which suggests an epiphytotic situation in Ukrainian apple plantations (Table 1).

Table 1. Prevalence of viral infections in apple plantations in Ukraine

Virus	Number of positive samples	Overall prevalence (%)	Prevalence among infected samples (%)	Share of monoinfections (%)	Percentage of mixed infections (%)	Asymptomatic infections (%)
ASPV	161	46.0	65.2	58.4	41.6	38.5
ACLSV	126	36.0	51.0	42.9	57.1	42.9
ApMV	48	13.7	19.4	35.4	64.6	29.2
Total*	247	70.6	100.0	-	-	41.7

Notes: * – general indicators consider samples infected with at least one virus
Source: compared by the authors based on the conducted research using the method of P.N. Bogner & A.A. Killeen (2006)

A detailed analysis revealed a clear differentiation in the prevalence of individual pathogens (Table 1). The highest prevalence was demonstrated by ASPV, which was identified in 161 samples, which is 46.0% of the total number of samples tested. ACLSV was the second most common virus – it was detected in 126 samples, which corresponds to 36.0% of the total number. The least common among the pathogens studied was ApMV, which was detected in only 48 samples, which was 13.7%. Attention is drawn to the fact that among the infected samples, only 58.3% showed any visible symptoms of the disease, while 41.7% of infected plants did not have any visual signs of damage, which indicates a significant spread of latent forms of infection. The highest percentage of asymptomatic infections was observed for ACLSV (42.9%), while for ASPV this figure was 38.5%, and for ApMV – 29.2%. To confirm the specificity of the results obtained, Sanger sequencing was performed (Crossley *et al*., 2020) on an analyser for 45 randomly selected amplicons (15 for each virus). The obtained sequences showed 98-100% homology with the reference virus strains, which confirmed the high specificity of the primer systems used and the reproducibility of PCR analysis results.

As shown in Table 1, ASPV is not only the most common virus in apple plantations, but also characterised by the highest frequency of monoinfections (58.4%). In contrast, ApMV is significantly more often detected in mixed infections (64.6%), which may indicate its lower competitiveness in individual plant damage. ACLSV occupies an intermediate position, but is characterised by the highest percentage of asymptomatic infections (42.9%), which makes it dangerous from the standpoint of uncontrolled spread through planting material. The results obtained indicate a critical situation with the phytosanitary condition of apple plantations in Ukraine and the need for urgent introduction of a system of certification of planting material based on sensitive molecular diagnostic methods.

The high overall infection rate of apple plantations, which was 70.6%, is a negative indicator that exceeds data from many other regions. The data obtained in the course of the study empirically confirm the conclusions of the EFSA Panel on Plant Health *et al*. (2021) on the risks of spreading viruses through planting material from Ukraine. In particular, the detected high level of latent infections (31.2% of all infected samples) without clinical manifestations justifies these data, since it creates a real threat of uncontrolled spread of pathogens through visually healthy planting material. The highest rate of asymptomatic infections detected for ACLSV – 42.9%, is dangerous due to its high prevalence (36.0%) in the plantings under study. These results highlight the lack of a visual method for assessing the condition of planting material and the need to introduce mandatory molecular testing. The dominance of ASPV virus (46.0%) in Ukrainian plantings is consistent with the results of studies in other countries, although at different levels. For example, W. Shi *et al.* (2020) reported a 35% prevalence of ASPV in China, while C. Canales *et al*. (2021) detected this virus in 42% of samples in Spain. This sequence may indicate the universality of the epidemiological properties of ASPV and its ability to effectively adapt to different climatic conditions. The high prevalence of ACLSV (36.0%) was also confirmed by other researchers. K. Zikeli *et al.* (2025) recorded

similar trends in Germany, although at a lower level (28%), which is associated with a more efficient system of certification of planting material. The results obtained demonstrate the state of viral damage to apple plantations in Ukraine, which is characterised by a high overall infection rate (70.6%), the dominance of the ASPV (46.0%), and a significant proportion of latent infections (41.7%), which makes it necessary to introduce a molecular monitoring system. Investigation of the regional features of the spread of viral infections will allow detailing this picture and developing targeted measures to improve phytosanitary control in various horticultural areas of the country.

Regional features of the spread of viral infections in the areas under study

A detailed regional analysis revealed statistically significant differences in the prevalence of viruses between the studied regions of Ukraine ($p < 0.01$ according to the χ^2 criterion), which indicates the influence of the geographical factor on the epidemiology of viral diseases of apple trees. The study covered five key regions (Kyiv, Vinnytsia, Cherkasy, Kharkiv, and Zakarpattia), which represent different agroclimatic zones of the country and have differences in the intensity of horticulture, cultivation technologies, and the history of the industry. The data obtained are presented in Table 2.

Table 2. Regional distribution of viral infections of apple trees in Ukraine

Area	Total number of samples	Total infection rate (%)	ASPV (%)	ACLSV (%)	ApMV (%)	Mixed infections (%)	Asymptomatic infections (%)
Zakarpattia	50	86.0	62.0	48.0	18.0	34.0	25.6
Kyiv	80	76.3	50.0	41.3	15.0	28.8	38.3
Vinnytsia	80	72.5	44.8	38.1	12.5	22.5	32.8
Cherkasy	70	67.1	41.4	32.9	11.4	18.6	35.4
Kharkiv	70	56.8	34.3	25.7	8.6	14.3	42.5

Source: compared by the authors based on the conducted research using the methods of Z. Ji *et al*. (2013), L. Hao *et al*. (2016)

The highest overall infection rate was recorded in orchards in the Zakarpattia region, where viruses were detected in 43 out of 50 samples, which is 86.0% of the total number of tested samples in this region. In this area, absolute dominance of the ASPV was observed, which was detected in 31 samples (62.0%). ACLSV also showed high prevalence rates – 24 positive samples (48.0%), while ApMV was detected in 9 samples (18.0%). A special feature of the Zakarpattia region was the highest proportion of mixed infections among all regions – 34.0% of the total number of samples. In the Kyiv region, the overall infection rate was 76.3% (61 out of 80 samples), with a clear predominance of ASPV (40 samples, 50.0%) and ACLSV (33 samples, 41.3%). ApMV in this region was detected in only 12 samples (15.0%). For the Kyiv region, a characteristic feature was a high proportion of asymptomatic infections – 38.3% of all infected samples, which may indicate a long-term circulation of viruses in the region and adaptation of the pathosystem. In the Vinnytsia region, viruses were detected in 58 out of 80 samples (72.5%), with a predominance of ASPV (44.8%) and ACLSV (38.1%). ApMV continued to show relatively low rates of 12.5%. This region was characterised by an average proportion of mixed infections (22.5%) and a moderate rate of asymptomatic infections (32.8%). In Cherkasy region, the total infection rate was 67.1% (47 out of 70 samples), with a prevalence of ASPV of 41.4% and ACLSV of 32.9%. ApMV was detected in 11.4% of the samples. This region was characterised by the lowest frequency of mixed infections among all the regions under study (18.6%). The lowest infection rates were recorded in the Kharkiv region – 40 out of 70 samples (56.8%). In this region, ASPV was detected in 34.3% of samples, ACLSV in 25.7%, and ApMV in only 8.6%. However, the highest proportion of asymptomatic infections was observed was in the Kharkiv region (42.5%), which may indicate a relatively recent penetration of viruses into the region or the specifics of local varieties.

Statistical analysis revealed a clear geographical pattern: the level of infection decreased in the direction from the west to the east of the country. Such regional differences are related to a complex of factors, including climatic features (higher

humidity in the western regions contributes to the development of virus vectors), the level of gardening intensity (older gardens in the western regions), the effectiveness of quarantine measures and the quality of planting material used in different regions of the country. The data obtained are of practical importance for the development of differentiated regional strategies for protecting apple trees from viral infections.

L. Yin *et al.* (2021) also found similar regional differentiation in Canada, linking it to climatic conditions and the age of plantings. In this case, higher humidity and older gardens in western regions can contribute to the activation of virus vectors and their accumulation over time. The results obtained regarding the high prevalence of viral infections of apple trees in Ukraine are confirmed in modern international studies. In particular, H. Xiao *et al.* (2022) in the study of the viromics of apple orchards in Canada also found a high level of infection, linking this to the phenomenon of "rapid decline" of orchards. However, Ukrainian figures (70.6%) exceed Canadian data, which may indicate a more intensive circulation of pathogens in Ukraine. Thus, the regional analysis revealed a clear geographical pattern of the spread of viral infections of apple trees in Ukraine with a significant differentiation between Western (86.0% in Zakarpattia) and Eastern (56.8% in Kharkiv) regions, which is conditioned by a complex of climatic, technological, and historical factors and requires the development of differentiated regional approaches to the protection of plantings. Studying the features of mixed infections will help to better understand the mechanisms of interaction of various viral pathogens in Ukrainian agrocoenoses.

Characteristics of mixed viral infections of apple trees in Ukraine

A detailed analysis of the detected viral infections showed a high proportion of mixed infections that were recorded in 89 samples, which is 25.4% of the total number of tested samples or 36.0% of all infected plants. This indicator suggests a significant accumulation of viral pathogens in Ukrainian apple plantations and indicates the complex nature of the epidemiological situation. As shown in Table 3, the combination of ASPV + ACLSV is absolutely dominant among all types of mixed infections, accounting for more than two-thirds of all cases of multiple lesions. The regional distribution indicates a clear geographical specificity – Zakarpattia and Kyiv regions show the highest prevalence of mixed infections, which may be due to climatic features and the intensity of horticulture in these regions.

Table 3. Characteristics of mixed viral infections of apple trees in Ukraine

Type of infection	Number of samples	Overall prevalence (%)	Prevalence among mixed infections (%)	Predominant symptoms	Most affected regions
ASPV + ACLSV	64	18.3	71.9	Leaf deformity, chlorosis, reduced growth	Zakarpattia (34.0%), Kyiv (28.8%)
ASPV + ApMV	15	4.3	16.9	Mosaic, vein necrosis, fruit deformity	Vinnytsia (6.3%), Cherkasy(4.3%)
ACLSV + ApMV	7	2.0	7.9	Chlorosis, mosaic, leaf crushing	Kyiv (3.8%), Kharkiv (2.9%)
ASPV + ACLSV + ApMV	3	0.9	3.4	Severe deformity, necrosis, dwarfism	Zakarpattia (2.0%)

Source: compared by the authors based on the conducted research using the methods of Z. Ji *et al*. (2013), L. Hao *et al*. (2016)

The most common among mixed infections was a combination of ASPV and ACLSV, which was detected in 64 samples, which is 18.3% of the total number of analysed samples. This combination prevailed in all regions under study, which may indicate a synergistic interaction between the two viruses or similarity in their epidemiological cycles. Dual ASPV + ApMV infections were reported in 15 samples (4.3%), and the ACLSV + ApMV combination was detected in 7 samples (2.0%).

Attention is drawn to the detection of a triple infection that included all three viruses under study, which was identified in 3 samples (0.9%). All these samples were obtained from the Zakarpattia region and showed pronounced symptoms in the form of severe deformation of the leaf plate, necrotic spots along the veins, and general growth inhibition. The intensity of symptoms in these cases significantly exceeded the manifestations of individual infections, which confirms the concept of a synergistic effect in mixed viral lesions.

Correlation analysis revealed a statistically significant positive association between the age of plantings and the frequency of mixed infections ($r = 0.72$, $p < 0.05$), which indicates the cumulative nature of the accumulation of viral infections in gardens over time. A high level of mixed infections was registered in plantings over 15 years of age, where this figure reached 42.7%. In young plantings (up to 5 years), mixed infections were detected much less often – only in 12.3% of samples. Regional analysis showed that the highest frequency of mixed infections was observed in the Zakarpattia region (34.0%), while the lowest rates were recorded in Kharkiv (14.3%) and Cherkasy (18.6%) regions. This pattern correlates with the overall level of infection in these regions and is related to the climatic conditions that contribute to the development of virus vectors and the duration of horticulture in these regions. A notable result is that mixed infections were accompanied by significantly more pronounced infection symptoms compared to monoinfections. In samples with mixed infections, the intensity of symptoms was 45-60% higher, and the expression of pathological signs began on average 2-3 weeks earlier during the growing season.

A high rate of mixed infections (25.4%), namely ASPV + ACLSV combinations (18.3%), is a significant outcome. A.A. Wright *et al.* (2020) also found that it is these combinations that lead to a synergistic effect and a significant increase in pathogenesis. Current observations of increased symptoms in mixed infections fully support this concept. It is significant that all cases of triple infections were detected in the Zakarpattia region and were accompanied by the most pronounced symptoms. A useful aspect is comparison with studies of related cultures. N.-Y. Kim *et al.* (2021) found similar patterns of pathogen spread when studying pear viruses using RNA-seq, which confirms the universality of epidemiological processes in fruit crops of the family Rosaceae. The high proportion of mixed infections should be noted, which is observed in both apple and pear trees. Another aspect is the comparison of the results obtained with studies in other cultures. For example, A. Karanfil (2021), when studying rose viruses, also found a high rate of mixed infections, which may indicate general patterns of virus spread in perennials. The study by F. Xing *et al*. (2020) for ornamental crop viruses revealed similar patterns of pathogen spread. The researchers conducted molecular characterisation of isolates of the Prunus necrotic ringspot virus from roses, revealing high genetic diversity. This confirms the universality of the patterns identified in this study and the need to develop a unified strategy for combating viral diseases in horticulture in Ukraine.

Varietal differences in susceptibility to viral infections

A detailed analysis of varietal sensitivity revealed differences in the susceptibility of the studied apple varieties to viral infections, which indicates the influence of genetic factors on the plant-pathogen interaction. The study covered four commercially significant varieties that occupy leading positions in industrial horticulture in Ukraine: 'Golden Delicious', 'Idared', 'Gala', and 'Champion' (Table 4).

Table 4. Varietal features of apple tree viral infections in Ukraine

Variety	Total infection rate (%)	ASPV infection rate (%)	ACLSV infection rate (%)	ApMV infection rate (%)	Frequency of mixed infections (%)	Preferred type of infection
'Golden Delicious' (n = 100)	78.4	54.3	41.6	15.2	32.1	ASPV + ACLSV (22.4%)
'Idared' (n = 90)	74.2	48.7	41.6	12.9	27.3	ASPV + ACLSV (18.9%)

Table 4. Continued

Variety	Total infection rate (%)	ASPV infection rate (%)	ACLSV infection rate (%)	ApMV infection rate (%)	Frequency of mixed infections (%)	Preferred type of infection
'Gala' (n = 80)	67.8	42.1	35.2	11.4	23.8	Uniform distribution
'Champion' (n = 80)	63.5	36.8	32.1	9.8	18.9	ASPV monoinfection (42.1%)

Source: compared by the authors based on the conducted research using the methods of Z. Ji *et al*. (2013), L. Hao *et al*. (2016)

The highest overall susceptibility to viral infections was demonstrated by the 'Golden Delicious' variety, in which the infection rate was 78.4% of all tested samples. This indicator significantly exceeded the average values for the entire sample. The second most affected variety was 'Idared' with an infection rate of 74.2%. Significantly lower rates were observed in the varieties 'Gala' (67.8%) and 'Champion' (63.5%), which indicates their relatively higher resistance to viral pathogens.

Statistical analysis using the χ^2 criterion confirmed the significance of varietal differences in susceptibility to ASPV at a significance level of $p < 0.05$. The 'Golden Delicious' variety showed the highest sensitivity to ASPV (54.3% of infected samples), while the 'Champion' variety showed only 36.8%. For ACLSV and ApMV, varietal differences did not reach statistical significance, although the trend towards higher susceptibility of 'Golden Delicious' and 'Idared' varieties persisted.

Analysis of the structure of infections in different varieties is of interest. The 'Golden Delicious' variety was characterised by a high frequency of mixed infections, which accounted for 32.1% of all infected samples of this variety. ASPV + ACLSV combinations prevailed (22.4%), while triple infections occurred in 3.7% of cases. In contrast, monoinfections prevailed for the 'Champion' variety (71.4% of all infected samples of the variety), with ASPV monoinfection being the most common (42.1%). The 'Idared' variety showed intermediate characteristics with a frequency of mixed infections of 27.3%, but with a high susceptibility to ACLSV (41.6%). The 'Gala' variety was characterised by the most equal distribution of infection types and a moderate frequency of mixed lesions (23.8%).

It was noted that varietal differences were manifested not only in the frequency of infection, but also in the nature of clinical manifestations. In the 'Golden Delicious' variety, even monoinfections were accompanied by pronounced symptoms – intense chlorosis and deformation of the leaf plate. In the 'Champion' variety, even mixed infections occurred with minimal manifestations, which indicates the presence of effective tolerance mechanisms. Regional analysis revealed no significant differences in varietal sensitivity between different regions of Ukraine, which confirms the stability of genetically determined susceptibility regardless of growing conditions.

Correlation between symptoms and molecular diagnostic results

Comparative analysis of the results of molecular diagnostics and phytopathological observations revealed a complex and multidimensional interaction between visual symptoms and the presence of specific viral infections. Among samples with severe leaf mosaic symptoms that were characterised by typical uneven leaf plate colour with alternating light green and yellow areas, ApMV was detected only in 58.3% of cases. This fact indicates the significant involvement of other pathogens in the development of similar symptoms, in particular, some strains of ASPV and individual phytoplasmas. In approximately 23.5% of cases, mosaicism was accompanied by simultaneous detection of two or more viruses, which makes it difficult to diagnose aetiology solely based on visual observations.

In samples with symptoms of chlorosis that manifested as inter-vein yellowing or lightening along the veins, ACLSV was detected in 72.4% of cases. This confirms the key aetiological role of this pathogen in the development of this

symptom complex. However, it should be noted that the intensity of chlorosis correlated with the type of infection: in mixed ACLSV + ASPV infections, symptoms were significantly more pronounced and spread over a larger area of the leaf surface. The highest correlation between the presence of symptoms and virus detection was observed for ASPV. Among samples with characteristic leaf deformity, including twisting, leaf plate asymmetry, threadiness and wavy edges, this pathogen was detected in 84.6% of cases. This high specificity makes leaf deformity a relatively reliable diagnostic marker for preliminary identification of ASPV infection in the field.

Another result of the study is the detection of a significant proportion of asymptomatic infections – 31.2% of all infected samples did not show any visible symptoms of the disease. This confirms the widespread prevalence of latent forms of infection, which is particularly dangerous for the spread of viruses through planting material. The highest percentage of asymptomatic infections was observed for ACLSV (42.9%), which is associated with the long co-evolution of this virus with apple trees and the development of mechanisms for "mitigating" pathogenesis. For ASPV, this figure was 38.5%, and for ApMV – 29.2%.

Correlation analysis revealed a weak negative correlation between symptom intensity and altitude ($r = -0.38$, $p < 0.05$). In areas located at an altitude of more than 300 metres above sea level (foothill areas of Zakarpattia and Vinnytsia regions), the intensity of symptoms was on average 25-30% lower compared to flat areas, despite the same level of infection. This indicates the influence of climatic factors, in particular, lower night temperatures and high humidity, on the expression of viral diseases. Significant varietal differences in the severity of symptoms in similar viral infections were established. For example, in the 'Golden Delicious' variety, even ACLSV monoinfection caused intense chlorosis, while in the 'Champion' variety, even mixed infections were often almost asymptomatic. This indicates a significant influence of host genetic factors on the pathogenesis of viral infections.

The use of RT-PCR has demonstrated its effectiveness for mass screening, but recent studies show that the introduction of the latest diagnostic methods is promising. In particular, J. Jiao *et al.* (2020) proposed CRISPR/Cas12a, a system for visual virus detection that can significantly speed up and simplify the testing process. Similarly, L. Yin *et al.* (2021) described the prospects of CRISPR-Cas systems for diagnosing plant viruses. The introduction of such methods in Ukrainian practice could significantly increase the effectiveness of phytosanitary control. Methodological aspects of the study indicate the need to introduce advanced approaches to the diagnosis of apple tree viruses in Ukraine. The results obtained, in particular, the high level of asymptomatic infections (31.2%) and regional features of the spread of pathogens, justify the feasibility of developing rapid test systems based on the experience of S. Franco Ortega *et al*. (2020), who developed innovative molecular methods for rapid detection of apple scab using isothermal amplification.

CRISPR/Cas methods described by S. Srivastava *et al.* (2020) demonstrated high sensitivity in detecting various plant viruses. Remote sensing technologies by Y.M. Wang *et al.* (2022) proposed an innovative approach to detecting viral infections using spectral analysis. The need for a combined diagnostic approach was emphasised by A. Kanapiya *et al.* (2024), supported by current data on the high frequency of mixed infections (25.4%). A notable aspect is the genetic characteristics of viruses. M.S. Noorani *et al.* (2023) emphasised the importance of full-genome sequencing for accurate identification of strains, which is important for Ukrainian isolates due to their high genetic diversity. The emergence of new viral pathogens, such as *Apple necrotic mosaic virus*, detected by S.U. Nabi *et al.* (2020) in India, pointed out the need for continuous monitoring of the viral spectrum in Ukrainian plantings. Viral disease management strategies proposed by A. Mandal *et al.* (2024) can be adapted to Ukrainian conditions. An important aspect is the implementation of LAMP methods similar to those developed by H.J. Lee & R.D. Jeong (2022) for ASPV detection. The economic aspect of viral infections requires a separate study, as evidenced by N. Mohammadi *et al*. (2023) on the commercial value of processed apple products.

Thus, the results obtained not only state the difficult situation with viral diseases of apple trees in Ukraine, but also lay the foundations for the introduction of advanced diagnostic methods and the development of an effective system of

phytosanitary control, which forms the basis for further research and practical measures to improve the condition of apple plantations in Ukraine. The results of the study on mixed viral infections of apple trees in Ukraine demonstrate a complex structure of multiple infections with a predominance of the combination of ASPV + ACLSV (71.9% of all mixed infections), accompanied by pronounced regional differentiation and increased pathological symptoms. The revealed specificity of interaction of viral pathogens in mixed infections, in particular, the synergistic effect in the combination of ASPV + ACLSV, which is manifested by increased deformation of leaves and chloroses, makes it necessary to develop targeted treatment strategies for different regions of Ukraine, considering local characteristics of the pathogenic complex.

CONCLUSIONS

The conducted study established a high level of infection of apple plantations in Ukraine with the main viral pathogens, which is 70.6% of the total number of samples analysed. This situation indicates the epiphytotic nature of the spread of viral diseases and makes it necessary to improve the system of phytosanitary control. Among some types of viruses, a clear hierarchy of prevalence was found: the most common was *Apple stem pitting virus* with an indicator of 46.0%, followed by *Apple chlorotic leaf spot virus* – 36.0%, and *Apple mosaic virus* – 13.7%. This dynamic is fully consistent with global trends, which confirms the universality of the epidemiological properties of these pathogens.

The study also revealed regional differences in the prevalence of viral infections in Ukraine. The highest infection rates were recorded in Zakarpattia (86.0%) and Kyiv (76.3%) regions, while the lowest – in Kharkiv (56.8%). Such regional disparities are caused by a complex of factors, including climatic features, age structure of plantings, intensity of horticulture, and effectiveness of phytosanitary measures in different regions. Attention is drawn to the high rate of mixed infections, which accounted for 25.4% of the total number of infected samples. The predominant combination was ASPV + ACLSV (18.3%), and the presence of a synergistic effect in such mixed infections was proved, which is manifested in a significant increase in pathogenesis and more pronounced symptoms of the disease. In addition, significant varietal differences in susceptibility to viral infections were established, where the most sensitive varieties were 'Golden Delicious' (78.4%) and 'Idared' (74.2%), and the most persistent – 'Gala' (67.8%) and 'Champion' (63.5%).

The study found a high incidence of asymptomatic infections, accounting for 31.2% of all infected samples. The highest rates of asymptomatic course were observed for ACLSV (42.9%), which poses a threat to the uncontrolled spread of viruses through planting material and significantly complicates effective phytosanitary control. The method of molecular diagnostics based on RT-PCR developed and tested during the study demonstrated its effectiveness for mass screening and can be recommended for introduction into the practice of phytosanitary control. The specificity of the results obtained was confirmed by sequencing the selected isolates, which proves the reliability of the approach used. A limitation of this study was that it covered only three major apple tree viruses and did not include analysis of other potentially important pathogens, such as *Apple stem grooving virus* or *Apple necrotic mosaic virus*, which could have led to an incomplete assessment of the phytosanitary condition of the plantings. Prospects for further research include expanding the spectrum of detected viruses, investigating the genetic diversity of Ukrainian isolates, introducing advanced diagnostic methods, and developing a strategy for integrated protection against viral diseases.

ACKNOWLEDGEMENTS

None.

FUNDING

None.

CONFLICT OF INTEREST

None.

REFERENCES

[1] Barros, H.L., Moresco, G., & Stefani, V. (2018). A simple protocol for the isolation, quantification and quality assessment of DNA and RNA. *Revista Virtual de Quimica*, 10(5), 1119-1126. doi: 0.21577/1984-6835.20180079.

[2] Bogner, P.N., & Killeen, A.A. (2006). Extraction of nucleic acids. In W.B. Coleman & G.J. Tsongalis (Eds.), *Molecular diagnostics: For the clinical laboratorian* (pp. 25-30). Totowa: Humana Press. doi: 0.1385/1-59259-928-1:025.

[3] Canales, C., Morán, F., Olmos, A., & Ruiz-García, A.B. (2021). First detection and molecular characterisation of *Apple stem grooving virus*, *Apple chlorotic leaf spot virus*, and *Apple hammerhead viroid* in loquat in Spain. *Plants*, 10(11), article number 2293. doi: 10.3390/plants10112293.

[4] Convention on Biological Diversity. (2025). Retrieved from https://www.cbd.int/convention/.

[5] Crossley, B.M., Bai, J., Glaser, A., Maes, R., Porter, E., Killian, M.L., Clement, T., & Toohey-Kurth, K. (2020). Guidelines for Sanger sequencing and molecular assay monitoring. *Journal of Veterinary Diagnostic Investigation*, 32(6), 767-775. doi: 0.1177/1040638720905833.

[6] Deben, C., Zwaenepoel, K., Boeckx, C., Wouters, A., Pauwels, P., Peeters, M., Lardon, F., Baay, M., & Deschoolmeester, V. (2013). Expression analysis on archival material revisited: Isolation and quantification of RNA extracted from FFPE samples. *Diagnostic Molecular Pathology*, 22(1), 59-64. doi: 10.1097/PDM.0b013e318269de3b.

[7] EFSA Panel on Plant Health (PLH), *et al*. (2021). Commodity risk assessment of Malus domestica plants from Ukraine. *EFSA Journal*, 19(11), article number e06909. doi: 0.2903/j.efsa.2021.6909.

[8] Franco Ortega, S., Prencipe, S., Gullino, M.L., & Spadaro, D. (2020). New molecular tool for a quick and easy detection of apple scab in the field. *Agronomy*, 10(4), article number 581. doi: 0.3390/agronomy10040581.

[9] Gritsenko, D.A., Aubakirova, K.P., Voitsekhovskiy, I., Soldatova, I., & Galiakparov, N.N. (2020). Simultaneous detection of five apple viruses by RT-PCR. *International Journal of Biology & Chemistry*, 13(1). doi: 0.26577/ijbch.2020.v13.i1.13.

[10] Hao, L., Xie, J., Chen, S., Wang, S., Gong, Z., Ling, K.-S., Guo, L., Fan, Z., & Zhou, T. (2016). A multiple RT-PCR assay for simultaneous detection and differentiation of latent viruses and apscarviroids in apple trees. *Journal of Virological Methods*, 234, 16-21. doi: 0.1016/j.jviromet.2016.04.003.

[11] Heo, S., & Chung, Y.S. (2020). Development of real-time quantitative PCR assay based on SYBR Green I and TaqMan probe for detection of apple viruses. *The Korean Journal of Crop Science*, 65(4), 496-507. doi: 0.7740/kjcs.2020.65.4.496.

[12] Ji, Z., Zhao, X., Duan, H., Hu, T., Wang, S., Wang, Y., & Cao, K. (2013). Multiplex RT-PCR detection and distribution of four apple viruses in China. *Acta Virologica*, 57(4), 435-441. doi: 0.4149/av_2013_04_435.

[13] Jiao, J., *et al*. (2020). Field detection of multiple RNA viruses/viroids in apple using a CRISPR/Cas12a-based visual assay. *Plant Biotechnology Journal*, 19(2), 394-405. doi: 0.1111/pbi.13474.

[14] Kairova, G., Daulet, N., Solomadin, M., Sandybayev, N., Orkara, S., Beloussov, V., Kerimbek, N., Gritsenko, D., & Sapakhova, Z. (2023). Identification of apple varieties resistant to fire blight (*Erwinia amylovora*) using molecular markers. *Horticulturae*, 9(9), article number 1000. doi: 0.3390/horticulturae9091000.

[15] Kanapiya, A., Amanbayeva, U., Tulegenova, Z., Abash, A., Zhangazin, S., Dyussembayev, K., & Mukiyanova, G. (2024). Recent advances and challenges in plant viral diagnostics. *Frontiers in Plant Science*, 15, article number 1451790. doi: 0.3389/fpls.2024.1451790.

[16] Karanfil, A. (2021). Prevalence and molecular characterization of Turkish isolates of the rose viruses. *Crop Protection*, 143, article number 105565. doi: 0.1016/j.cropro.2021.105565.

[17] Kerimbek, N., Kapytina, A.I., & Taskuzhina, A.K. (2024). Assessment of occurrence and diversity of apple viruses in South Kazakhstan. *Science and Education*, 1(2(75)), 79-85. doi: 0.52578/2305-9397-2024-2-2-79-85.

[18] Kim, N.-Y., Lee, H.J., Kim, H.S., Lee, S.H., Moon, J.S., & Jeong, R.D. (2021). Identification of plant viruses infecting pear using RNA sequencing. *The Plant Pathology Journal*, 37(3), 258-267. doi: 0.5423/PPJ.OA.01.2021.0009.

[19] Kwon, Y.H., Lee, G.R., Eom, H., Kim, H.K., Yoo, S.E., & Park, S. (2024). Efficacy of antiviral agents and thermotherapy in eliminating viruses from in vitro plantlets derived from apical meristems of fire blight resistant apple rootstocks G11 and G30. *Korean Journal of Plant Resources*, 37(6), 579-588. doi: 10.7732/kjpr.2024.37.6.579.

[20] Lee, H.J., & Jeong, R.D. (2022). A reliable reverse transcription loop-mediated isothermal amplification assay for detecting *Apple stem grooving virus* in pear. *Research in Plant Disease (식물병연구)*, 28(2), 92-97.
[21] Ma, L., *et al*. (2021). Incidence of major pome fruit tree viruses and viroids in commercial pear orchards in China and in Pyrus betulifolia seedlings. *Plant Pathology*, 70(6), 1467-1475. doi: 0.1111/ppa.13375.
[22] Mandal, A., Mukherjee, A., & Bandyopadhyay, R. (2024). Plant virus disease management strategies: Conventional versus modern techniques. In *Detection and management of new and emerging mystery plant virus sources* (pp. 207-238). New York: Apple Academic Press.
[23] Mishchenko, L.T., Dunich, A.A., Mishchenko, I.A., Dashchenko, A.V., Kozub, N.O., Kyslykh, T.M., & Molodchenkova, O.O. (2022). Wheat dwarf virus in Ukraine: Occurrence, molecular characterization and impact on the yield. *Journal of Plant Diseases and Protection*, 129(1), 107-116. doi: 0.1007/s41348-021-00552-w.
[24] Mohammadi, N., Guo, Y., Wang, K., & Granato, D. (2023). Macroporous resin purification of phenolics from Irish apple pomace: Chemical characterization, and cellular antioxidant and anti-inflammatory activities. *Food Chemistry*, 437, article number 137815. doi: 0.1016/j.foodchem.2023.137815.
[25] Nabi, S.U., Baranwal, V.K., Yadav, M.K., & Rao, G.P. (2020). Association of *Apple necrotic mosaic virus* (ApNMV) with mosaic disease in commercially grown cultivars of apple (*Malus domestica* Borkh) in India. *3 Biotech*, 10, article number 122. doi: 0.1007/s13205-020-2117-6.
[26] Noorani, M.S., Baig, M.S., Khan, J.A., & Pravej, A. (2023). Whole genome characterization and diagnostics of prunus necrotic ringspot virus (PNRSV) infecting apricot in India. *Scientific Reports*, 13, article number 4393. doi: 0.1038/s41598-023-31172-z.
[27] Pavliuk, L., Udovychenko, K., Riaba, I., & Bublyk, M. (2021). Detection of sour and sweet cherry viruses in Ukraine. *Agronomy Research*, 19(1), 199-209. doi: 0.15159/ar.20.238.
[28] Shi, W., Yao, R., Sunwu, R., Huang, K., Liu, Z., Li, X., Yi, Y., & Wang, J. (2020). Incidence and molecular identification of *Apple necrotic mosaic virus* (ApNMV) in Southwest China. *Plants*, 9(4), article number 415. doi: 0.3390/plants9040415.
[29] Srivastava, S., Upadhyay, D.J., & Srivastava, A. (2020). Next-generation molecular diagnostics development by CRISPR/Cas tool: Rapid detection and surveillance of viral disease outbreaks. *Frontiers in Molecular Biosciences*, 7, article number 582499. doi: 0.3389/fmolb.2020.582499.
[30] Tsvigun, V.O., Levishko, A.S., Gumeniuk, I.I., & Mazur, S.O. (2024). Monitoring of viral infections in vegetable crops in agroecosystems of Ukraine. *Microbiological Journal*, 86(5), 87-101. doi: 0.15407/microbiolj86.05.087.
[31] Wang, Y.M., Ostendorf, B., Gautam, D., Habili, N., & Pagay, V. (2022). Plant viral disease detection: From molecular diagnosis to optical sensing technology – a multidisciplinary review. *Remote Sensing*, 14(7), article number 1542. doi: 0.3390/rs14071542.
[32] Wright, A.A., Cross, A.R., & Harper, S.J. (2020). A bushel of viruses: Identification of seventeen novel putative viruses by RNA-seq in six apple trees. *PLoS One*, 15(1), article number e0227669. doi: 0.1371/journal.pone.0227669.
[33] Xiao, H., Hao, W., Storoschuk, G., MacDonald, J.L., & Sanfaçon, H. (2022). Characterizing the virome of apple orchards affected by rapid decline in the Okanagan and Similkameen valleys of British Columbia (Canada). *Pathogens*, 11(11), article number 1231. doi: 0.3390/pathogens11111231.
[34] Xing, F., Gao, D., Liu, H., Wang, H., Habili, N., & Li, S. (2020). Molecular characterization and pathogenicity analysis of prunus necrotic ringspot virus isolates from China rose (*Rosa chinensis* Jacq.). *Archives of Virology*, 165, 2479-2486. doi: 0.1007/s00705-020-04739-8.
[35] Yin, L., Man, S., Ye, S., Liu, G., & Ma, L. (2021). CRISPR-Cas based virus detection: Recent advances and perspectives. *Biosensors and Bioelectronics*, 193, article number 113541. doi: 0.1016/j.bios.2021.113541.
[36] Zikeli, K., Berwarth, C., Born, U., Leible, T., Jelkmann, W., & Hagemann, M.H. (2025). Prevalence, genetic diversity, and molecular detection of the apple hammerhead viroid in Germany. *Frontiers in Microbiology*, 16, article number 1592572. doi: 0.3389/fmicb.2025.1592572.

[37] Zuļģe, N., Gospodaryk, A., & Moročko-Bičevska, I. (2023). Genetic diversity and phylogenetic relationships of *Apple chlorotic leaf spot virus* isolates from Malus, Pyrus and Prunus hosts in Latvia. *Plant Pathology*, 72(5), 900-911. doi: 10.1111/ppa.13712.

Молекулярна ідентифікація та моніторинг основних вірусів яблуні в Україні

Катерина Гринчук

Кандидат біологічних наук, доцент
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
https://orcid.org/0000-0002-3138-0080

Ігор Антіпов

Кандидат сільськогосподарських наук, доцент
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
https://orcid.org/0000-0002-1156-267X

Анотація. Вірусні захворювання яблуні спричиняють значні економічні втрати в садівництві України, що потребує сучасного дослідження їх поширеності. Метою роботи було оцінити поширеність та регіональний розподіл основних вірусів яблуні у промислових насадженнях України за допомогою молекулярних методів діагностики. Дослідження проводили методом зворотної транскрипції та полімеразної ланцюгової реакції на 350 зразках листя та кори яблуні, відібраних із промислових насаджень п'яти областей України. Специфічність детекції трьох цільових вірусів підтверджували секвенуванням отриманих ампліконів. В ході дослідження встановлено, що загальний рівень інфікованості яблуневих насаджень склав 70,6 %, що свідчить про епіфітотичну ситуацію. Найпоширенішим виявився вірус ямчастості деревини яблуні (46,0 %), тоді як вірус хлоротичної плямистості листя яблуні та вірус мозаїки яблуні виявлені у 36,0 % та 13,7 % зразків відповідно. Регіональний аналіз виявив максимальну поширеність вірусів у Закарпатській (86,0 %) та Київській (76,3 %) областях, тоді як мінімальні показники зафіксовані в Харківській області (56,8 %). Встановлено високий рівень змішаних інфекцій (25,4 %), з переважанням комбінації вірусу ямчастості деревини яблуні та вірусу хлоротичної плямистості листя яблуні (18,3 %). Також значущим є високий відсоток безсимптомних інфекцій (31,2 %), що ускладнює фітосанітарний контроль. Висновки підтвердили, що отримані результати обґрунтовують необхідність термінового впровадження системи молекулярного моніторингу та сертифікації посадкового матеріалу. Результати дослідження можуть бути використані для розробки національної програми оздоровлення яблуневих насаджень України

Ключові слова: полімеразна ланцюгова реакція; вірус ямчастості деревини яблуні; вірус хлоротичної плямистості листя яблуні; вірус мозаїки яблуні

Received: 12.06.2025 Revised: 24.10.2025 Accepted: 27.11.2025 Published: 26.12.2025

BIOLOGICAL SYSTEMS: Theory and Innovation

ISSN 2706-8382
e-ISSN 2706-8390

Journal homepage: https://bioscience.com.ua/en
Vol. 16, No. 4, 2025, 71-89

UDC 579.2

DOI: 10.31548/biologiya/4.2025.71

Biotechnological aspects of the formation of the metagenome of potato rhizosphere microorganisms under the influence of microbial preparations

Vitalii Overchenko*

PhD in Agricultural Sciences, Associate Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
https://orcid.org/0009-0009-5435-6815

Yuliia Kolomiets

Doctor of Agricultural Sciences, Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
https://orcid.org/0000-0002-1919-6336

Abstract. The study aimed to assess the impact of microbial preparations on the structural and functional characteristics of the potato rhizosphere metagenome and the associated changes in plant growth and productivity. Within the framework of the vegetation experiment, whole-genome metagenomic profiles of the potato rhizosphere were obtained for five variants: untreated, three separate microbial preparations, and their combination. A total of 11.8 million sequencing reads were obtained, which underwent basic quality filtering; 2,950 taxonomic units were identified, taxonomic and functional annotation was performed, diversity indices were calculated, and a correlation analysis with morphological and yield indices was conducted. The study determined that representatives of the Proteobacteria, Actinobacteriota, Bacteroidota and Firmicutes types dominated in all variants, with a total share exceeding 80% of the metagenome structure, while the use of microbial preparations was accompanied by an increase in species richness and diversity: the Shannon index increased from 4.82 in the control to 5.12-5.18 in the experimental variants, and the Chao1 richness estimate increased from 830 to 960-975 taxa. An increase in the proportion of genera associated with plant growth was observed, as well as in the relative number of genes associated with nitrogen fixation, phosphorus mobilisation, potassium transport, sulphur assimilation and stress resistance, with maximum values in the variants with the third preparation and its combination with the first. These options ensured an increase in tuber weight per plant to 495-510 g and an increase in the proportion of medium and large fractions of the harvest, with correlation coefficients between the total proportion of beneficial microorganisms, functional genes and productivity reaching 0.6-0.7. The results obtained showed that targeted microbial treatment can form a rhizosphere metagenome with increased biotechnological potential and can be used by practising agronomists, developers of microbial preparations, potato breeders, and specialists in integrated soil fertility management to optimise microbial consortia in potato cultivation technologies

Keywords: variant; quantity; type; genus; nitrogen fixation; phosphorus

Suggested Citation:

Overchenko, V., & Kolomiets, Yu. (2025). Biotechnological aspects of the formation of the metagenome of potato rhizosphere microorganisms under the influence of microbial preparations. *Biological Systems: Theory and Innovation*, 16(4), 71-89. doi: 10.31548/biologiya/4.2025.71.

*Corresponding author

INTRODUCTION

The research relevance is determined by the need to improve methods of managing the rhizosphere microbiome of cultivated plants, in particular potatoes, to increase their productivity and resistance to stress factors. Modern agricultural technologies, such as biological preparations, genetically modified organisms (GMOs), robotisation and automation of agricultural processes, are aimed at integrating biological preparations containing beneficial microorganisms to optimise plant nutrition processes, stimulate their growth and improve soil fertility. Biological products can be used to improve the structure of the rhizosphere microbiome, which in turn affects the morphological and productive indicators of plants. Genetically modified plants improve resistance to pests and diseases, reducing the need for chemical agents. Robotisation and automation of agronomic processes reduce labour costs and improve the accuracy of operations, which contributes to more efficient crop cultivation. The use of biological products, in particular to improve the rhizosphere microbiome, has the potential to change the composition of microbial communities, which can lead to improved plant health and higher yields. Research into the metagenomic profile of rhizosphere microorganisms further contributes to the analysis of their functional potential, creating new opportunities for the introduction of innovative biotechnologies in agriculture and contributing to the sustainable development of agricultural production.

The effect of microbial preparations on the response of potato varieties to treatment with nitrogen-fixing bacteria and mycorrhiza was studied in a paper by V. Kovalenko *et al.* (2024). The study determined that the use of microbial preparations improves potato growth and productivity by promoting more efficient nitrogen use. A. Melnyk & M. Kyryk (2020) analysed the effectiveness of biological preparations in reducing potato alternaria in the forest-steppe zone of Ukraine. The study found that the use of biological preparations reduces disease incidence and improves crop quality, confirming the potential of biological agents in crop production. M.O. Kiroyants (2024) studied the formation of the barley rhizosphere microbiome under different fertiliser systems in typical chornozems. The study determined that the type of fertiliser affects the composition of microbial communities, which is relevant for an increase in soil fertility and crop yields.

Microbial diversity and dynamics of potato rhizosphere communities during cultivation were assessed in a study by Q. Hou *et al.* (2020). The study determined that the potato microbiome undergoes significant changes depending on agronomic factors, which affect its health and productivity. Additionally, the study demonstrated that the most pronounced changes in microbial communities occurred in the early stages of plant development, while after flowering, the structure of the microbiome became relatively more stable. P. Lu *et al.* (2023) studied the metagenomic analysis of the tobacco rhizosphere. The study demonstrated changes in microbial communities under the influence of root nematodes, confirming the influence of the microbiome on the regulation of plant stress resistance. The study also noted that the structure and functional potential of the rhizosphere bacterial community statistically correlated with the degree of damage by root nematodes. Changes in the microbial community in different underground compartments of potatoes, which affect crop yield and quality, were evaluated in a study by G. Chen *et al.* (2022). The study proved that changes in the microbiome can directly correlate with the agronomic characteristics of potatoes. A clear difference in microbiota between compartments (rhizosphere, tuber surface, soil) and regions was also shown, with soil nutrients and pH being the key factors of variation. A review of current metagenomic approaches to studying microbial diversity in the rhizosphere was conducted by B. Rajguru *et al.* (2024). The study considered the use of metagenomic technologies to assess the functional capabilities of the microbiome, which provides a complete overview of its impact on plants. The advantages and limitations of the approaches (shotgun metagenomics vs. 16S profiling) and their suitability for interpreting the functions of the microbiome, particularly in the context of nutrition and disease suppression, were systematised separately.

The assessment of the potato rhizosphere microbiome during bacteriophage therapy was conducted by S. Mousa *et al.* (2022). During the analysis, scientists discovered the possibility of modifying the microbial community to improve

plant health. Additionally, the study demonstrated that phage cocktails after infection with bacterial pathogens provided a better effect in reducing disease manifestations compared to monophages, confirming the controllability of rhizosphere microbiota as an element of biocontrol. N. Imam *et al.* (2021) determined that the properties of local microbiome networks in the soil are predictive factors for potato yield. This statement emphasises the role of the microbiome in agronomic performance. The study demonstrated that combining NGS data and bioinformatics with the Random Forest model provided high accuracy (84.6%) in distinguishing between low/high yield plots, with the network characteristics of fungal communities having higher predictive power than those of bacterial communities. The impact of different crop rotation systems on the potato rhizosphere microbiome was investigated by J. Qin *et al.* (2022). The study emphasised the significance of agronomic practices for microbiome management and yield improvement. Additionally, the study demonstrated that during long-term continuous cultivation, the incidence of diseases (in particular, black scurf) increased, and crop rotation and continuous cultivation regimes differently altered the diversity of bacterial and fungal communities in the soil and rhizosphere. Research into the microbial environment of the potato rhizosphere occupies a prominent place in agrobiotechnology, as the rhizosphere microbiome directly affects plant health, growth and productivity. Many authors have confirmed that changes in the composition of the microbial community can significantly improve the efficiency of agrochemicals and contribute to increasing plant resistance to diseases and stress factors. In particular, the use of biological preparations that stimulate the activity of beneficial microorganisms is a promising direction in agriculture for optimising the soil ecosystem and increasing crop yields. The study aimed to evaluate the effect of microbial preparations on the structural and functional characteristics of the potato rhizosphere metagenome. The objectives of the study were: 1) to analyse changes in the taxonomic composition of the rhizosphere microbiome under the influence of various microbial preparations; 2) to assess the relationship between changes in the microbiome and agronomic indicators of potatoes.

MATERIALS AND METHODS

The study was conducted in a field experiment with potatoes (*Solanum tuberosum* L.) of the mid-season variety 'Slovyanka' during the growing season from April to September 2025 in Ukraine, Kyiv region, in a temperate climate zone on sod-podzolic/chornozem soil (pH 6.2-6.5, humus content 2.5-3.0%). In addition, the agrochemical and agrophysical indicators of the soil were characterised, in particular the content of mineral nitrogen ($N\text{-}NO_3^-$, $N\text{-}NH_4^+$), mobile forms of phosphorus and potassium (P_2O_5, K_2O), micronutrient availability (Fe, Mn, Zn, Cu, B), as well as field moisture and soil temperature during vegetation (at the depth of the root-containing layer). The predecessor of potatoes was winter wheat, which provided a typical background for evaluating the effect of microbial preparations without excessive accumulation of organic residues of leguminous crops. The choice of crop (potatoes) and the 'Slovyanka' variety was justified by their prevalence in the forest-steppe zone of Ukraine, stable adaptability to the soil and climatic conditions of the Kyiv region, and suitability for comparative assessment of the effect of microbial inoculants on growth indicators, yield and rhizosphere microbiome parameters under typical technological conditions. The study included five variants (control, M1, M2, M3, M1+M2) and was conducted on plots of 10-12 m^2 for each variant, with a planting pattern of 70×25 cm, incorporating technological tracks and protective strips around the perimeter. Planting was conducted manually with calibrated tubers of standard size with visually healthy characteristics, to a depth of 6-8 cm, in pre-prepared soil without excessive compaction. Experimental studies of plants, including the collection of plant material, complied with institutional and national guidelines. The study adhered to the standards of the Convention on Biological Diversity (1992).

The experimental design consisted of five variants: control without treatment (C), three variants with separate microbial preparations (M1, M2, M3) and combined treatment with preparations M1+M2. In variant M1, the preparation "Azotofit" based on strains of nitrogen-fixing rhizobacteria *Azotobacter* spp. was used; in variant M2, the phosphate-mobilising Fosfoenter preparation based on *Bacillus* spp. and *Pseudomonas* spp. was

used; in variant M3, the complex biological Bio-Complex-Rhizo preparation was used, which included strains of *Rhizobium* spp., *Azospirillum* spp. and associated rhizobacteria capable of synthesising phytohormone-like compounds. In the combined variant M1+M2, these preparations were applied together in the same proportions as when used separately. The choice of these preparations was justified by their different PGPR mechanisms of action and the expected complementary effect on the rhizosphere microbiome and mineral nutrition of potatoes: M1 was chosen as an inoculant with the potential to increase nitrogen supply and stimulate root formation; M2 – as a preparation aimed at mobilising hard-to-reach forms of phosphorus and enhancing phosphorus nutrition; M3 – as a complex consortium combining nitrogen fixation, phosphate mobilisation and phytohormone-like growth stimulation. The combined option M1+M2 was included to test the possible synergistic effect of simultaneous enhancement of nitrogen and phosphorus nutrition and to assess whether the combination of functional groups of microorganisms leads to more pronounced changes in the taxonomic and functional structure of the rhizosphere microbiome compared to mono-inoculation. The microbial preparations belonged to a group of biological agents based on rhizosphere bacteria with a declared ability to fix nitrogen, mobilise phosphorus and synthesise biologically active metabolites. The treatment was conducted before planting by inoculating the tubers with working suspensions of the preparations in the concentrations recommended by the manufacturer, ensuring uniform application over the entire surface of each tuber (the same rate of working solution per unit of mass/number of tubers), with uniform wetting of the tubers and holding until drying, after which planting was conducted on the experimental plots.

The experiment was conducted using a randomised block design in three replicates, with each variant presented as a separate plot using standard potato cultivation techniques. For metagenomic analysis, five rhizosphere samples were selected from each variant (one sample from individual plants from different plots of the repetition), which provided 25 biological replicates. Samples from different replicates were not mixed but were processed and sequenced separately as independent biological replicates (each sample formed a separate library/separate data file for further taxonomic and functional analysis). Rhizosphere soil was collected during the period of active plant growth, after carefully extracting the tuberous plant and separating the soil adhering to the root system. The samples were immediately cooled in thermal containers with cooling elements (≈ +2...+6°C) and transported to the laboratory within 4-6 hours, where they were stored at -80°C until DNA (deoxyribonucleic acid) extraction. During sample preparation in the laboratory, standard microclimatic conditions were maintained (t 20-22°C; relative humidity 40-60%), which minimised the risk of condensation and secondary moistening of the samples.

Genomic DNA from rhizosphere soil was extracted using a soil sample kit (DNeasy PowerSoil Kit, QIAGEN, Germany) according to the manufacturer's instructions. The quality and concentration of DNA were monitored using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and a Qubit fluorometer (Thermo Fisher Scientific, USA). For metagenomic analysis, random fragmentation libraries were prepared and subsequently sequenced using paired-end technology on an Illumina platform (NovaSeq 6000, USA), which provided an average volume of approximately 0.49 million reads per sample.

Primary processing of sequencing data was performed using a standard pipeline, which included quality control, adapter sequence trimming, and quality threshold filtering. Taxonomic annotation was performed based on comparison with reference databases of bacterial genomes, forming OTUs (Operational Taxonomic Units)/ASVs (Amplicon Sequence Variants) and generalising them to the level of types, families and genera. The functional profile of the metagenome was formed based on the prediction of functions from 16S data using PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, version): predicted KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologs were aggregated into functional categories and metabolic pathways related to plant nutrition (nitrogen fixation, phosphorus mobilisation, potassium transport, sulphur assimilation) and stress resistance (biosynthesis of antibiotic-like compounds and siderophores, antioxidant enzymes,

resistance induction). The morphological and productive indicators of potatoes were determined on a representative sample of plants from each variant (at least 10 plants per repetition). Plant height was measured with a tape measure, tuber weight was measured on laboratory scales, and the number of tubers was measured by direct counting. The fractional composition of the harvest (small, medium, large tubers) was determined by weight and calibre, with the proportion of each fraction calculated as a percentage.

Intragroup diversity indices (Shannon, Simpson, Chao1) were calculated based on OTUs/ASVs tables. Intergroup differences in microbiome structures were assessed using Bray-Curtis taxonomic distance matrices followed by ordination (e.g., principal coordinates) and clustering of samples. Correlation analysis (Pearson's or Spearman's coefficients, depending on the data distribution) was used to analyse the relationships between microbiome indicators and productivity, controlling for the influence of external environmental factors (soil temperature and moisture) by partial correlation/inclusion of these indicators as covariates. The normality of the distribution was tested using the appropriate criterion, and differences between variants were assessed using parametric or non-parametric tests for independent samples with a statistical significance level of $p < 0.05$ and, if necessary, multiple comparison control. The integral indicator of biotechnological potential was calculated by normalising structural, functional and agronomic parameters to a dimensionless scale and then aggregating them into a single index with equal weights of blocks. All calculations were performed using specialised statistical and metagenomic analysis software packages.

RESULTS AND DISCUSSION

General characteristics of the metagenome of potato rhizosphere microorganisms

Within the scope of the study, whole-genome metagenomic profiles of rhizosphere samples of potatoes were obtained from five variants: control (C), treatment with microbial preparation 1 (M1), microbial preparation 2 (M2), microbial preparation 3 (M3) and combined treatment with preparations 1+2 (M1+M2). The total number of sequenced genera that passed the basic quality filtration was 11.8 million, with an average of 0.49 million genera per sample. In the control variant, 2.1 million genera were obtained, in variants M1, M2 and M3 – 2.4, 2.3 and 2.5 million genera, respectively, and in variant M1+M2 – 2.5 million genera. As a result of taxonomic annotation, 2,950 OTUs/ASVs were identified, belonging to 17 types, 46 classes, 118 families and 247 genera of prokaryotic microorganisms. In the control variant, the number of OTUs/ASVs averaged 820 ± 60 per sample, in variants M1, M2 and M3 – 910 ± 55, 880 ± 50 and 940 ± 65, respectively, and in variant M1+M2 – 960 ± 70 OTUs/ASVs per sample. The ratio between the number of genera and the number of OTUs/ASVs remained stable in most variants, reflecting a comparable level of taxonomic diversity coverage (Table 1).

Table 1. Summary indicators of sequencing and basic diversity of the potato rhizosphere metagenome

Variant	Number of births, million	Average number of OTUs/ASVs per sample	Observed richness, average, OTUs/ASVs	Shannon index, H′
K (control)	2.1	820	805	4.82
M1	2.4	910	905	5.05
M2	2.3	880	895	4.96
M3	2.5	940	935	5.12
M1+M2	2.5	960	945	5.18

Source: compiled by the authors based on research

Analysis of basic diversity indicators showed that the total number of taxa (OTUs/ASVs) at the sample and variant levels remained comparable, although an increase in species richness was observed in the variants with microbial preparations compared to the control. The mean values of observed richness at the OTUs/ASVs level were 805 for the control, 895-935 for individual

preparations, and 945 for the M1+M2 combination. Diversity indices that considered both richness and evenness (in particular, the Shannon index) ranged from 4.8 to 5.3 bits for most variants, with minimal differences between them (Table 1). The taxonomic spectrum of rhizosphere microbiomes in all variants was formed mainly by representatives of the Proteobacteria, Actinobacteriota, Bacteroidota, and Firmicutes types. The total share of these types in the overall metagenome structure ranged from 82.3% in the control to 87.5% in the M1+M2 variant. In the control variant, Proteobacteria (43.1%) and Actinobacteriota (22.4%) had the highest relative abundance, while in the variants with microbial preparations, the proportion of Proteobacteria ranged from 45.6 to 49.2% and Actinobacteriota from 20.1 to 23.8%. The proportion of Bacteroidota varied from 9.5% (control) to 11.7% (M3), and Firmicutes from 7.3% to 9.4%, depending on the variant (Fig. 1).

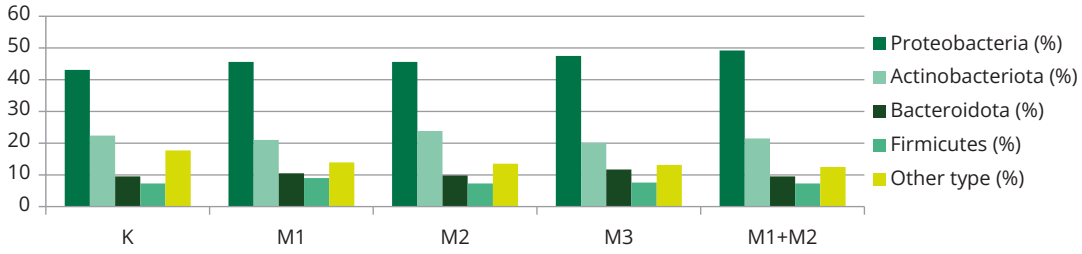

Figure 1. Relative abundance of dominant bacterial types in the potato rhizosphere under different microbial treatment options

Source: compiled by the authors based on research

This reflected a predominantly even distribution of abundance between dominant and subdominant taxa in all variants. A core microbiome of the potato rhizosphere was identified, consisting of taxa found in most samples regardless of variant. The core included representatives of the genera *Pseudomonas*, *Bacillus*, *Streptomyces*, *Flavobacterium*, *Rhizobium*, and *Sphingomonas*, whose total relative abundance was 41.2% in the control and 43.5-47.8% in the variants with microbial preparations. The proportion of core genera at the type level was distributed mainly between Proteobacteria (24.3-27.9%) and Actinobacteriota (10.8-12.6%).

The results of the study are consistent with the conclusions of J.K. Kiige *et al.* (2025), noting an increase in the diversity of the potato microbiome when treated with microbial preparations. Changes in the metagenome confirm the effect of such preparations on the stability of rhizosphere microbiomes. The study by M. Wang *et al.* (2025) also demonstrated the influence of agrotechnical conditions on the potato rhizosphere microbiome, which is reflected in the study on the influence of microbial preparations. In the variant with combined treatment with preparations, an increase in the proportion of genera that promote plant growth was noted. The results obtained in this work confirm the conclusions of J. Song *et al.* (2021) regarding the positive effect of biological preparations on the rhizosphere microbiome, which is associated with an improvement in the quality of potato tubers. In this study, as in the work of scholars, an increase in the diversity of the microbiome was accompanied by an improvement in agronomic indicators.

The generalisation of the results obtained made it possible to characterise the rhizosphere metagenome of potatoes as a taxonomically diverse bacterial community with a comparable level of sequencing depth between variants and a stable ratio of genera and OTUs/ASVs. In all variants, representatives of the Proteobacteria, Actinobacteriota, Bacteroidota and Firmicutes types dominated, accounting for more than 80% of the total metagenome structure, while the contribution of other types remained relatively limited. Variants with microbial preparations were characterised by higher species saturation compared to the control in terms of observed richness and average number of OTUs/ASVs per sample, while maintaining similar Shannon index values. The core microbiome of

the potato rhizosphere included the genera *Pseudomonas*, *Bacillus*, *Streptomyces*, *Flavobacterium*, *Rhizobium*, and *Sphingomonas*, which were present in most samples regardless of the variant, ensuring the stability of the basic taxonomic core when using different microbial preparations.

Dynamics of α-diversity of the rhizosphere microbiome under the influence of drugs

The indicators of intragroup diversity of the potato rhizosphere microbiome were assessed using the Shannon, Simpson, and Chao1 indices for all experimental variants (Table 2).

Table 2. α-diversity indices of the rhizosphere microbiome of potatoes

Variant	Shannon index, H′ (mean ± SD)	Simpson index (mean ± SD)	Chao1, OTUs/ASVs (average ± SD)
K (control)	4.82 ± 0.03	0.912 ± 0.003	830 ± 8.5
M1	5.05 ± 0.03	0.928 ± 0.003	940 ± 8.5
M2	4.96 ± 0.03	0.921 ± 0.003	925 ± 8.5
M3	5.12 ± 0.03	0.931 ± 0.003	960 ± 8.5
M1+M2	5.18 ± 0.03	0.935 ± 0.003	975 ± 8.5

Source: compiled by the authors based on research

A comparison of the experimental variants showed that the use of microbial preparations was accompanied by an increase in both taxon richness and the evenness of their relative abundance distribution compared to the control. The lowest values of Chao1, Shannon, and Simpson indices were recorded in the control variant, while all experimental variants were characterised by higher species saturation. The most pronounced increase in the assessed indicators was recorded in the combined treatment variant M1+M2, where the Chao1 and diversity index values exceeded those of the control and individual single-component treatments. In variants M1, M2 and M3, α-diversity also exceeded the control level, but the differences between individual preparations were less pronounced than between the control and combined treatment. Analysis of the richness (Chao1) and evenness (Shannon and Simpson indices) indices showed that the increase in α-diversity in the variants with microbial preparations was mainly due to an increase in the number of rare OTUs/ASVs while maintaining a high proportion of dominant taxa. Rare taxa were considered a "reservoir" of functional potential of the rhizosphere, since even at low relative abundance they may contain genes associated with key ecosystem functions (mobilisation of nutrients, synthesis of siderophores and antimicrobial metabolites, participation in hormone-like regulation and induction of systemic resistance). The increase in the proportion of rare OTUs/ASVs in the treatment groups was interpreted as a potential expansion of the functional niche of the microbiome and an increase in its functional redundancy, which may contribute to the stability of the microbial community and support plant productivity under changing environmental conditions. Elevated Chao1 values in the M3 and M1+M2 variants indicated a broader spectrum of taxa in the microbial community, while the increase in the Simpson index reflected a more even distribution of relative abundance among them. This combination of greater richness and evenness of the microbiome structure was considered an indicator of increased structural complexity and potential functional capacity of the rhizosphere community. In the context of microbial community stability, increased α-diversity values in the experimental variants, particularly in the combined treatment M1+M2, indicated the formation of a more diverse and evenly structured microbiome capable of supporting a wider range of metabolic functions due to the presence of both dominant and rare taxa. Within the scope of this study, this configuration of intragroup diversity was identified as the basis for further analysis of the relationship between α-diversity, functional characteristics of the metagenome, and agrobiological indicators of potato plants.

The results of the study of α-diversity of the potato rhizosphere microbiome are consistent with the study by J. Ha *et al.* (2021) also showed that treating plants with microbial preparations leads to an increase in microbiome diversity. Both studies observed an increase in the number of rare OTUs/ASVs, indicating an expansion of microbiological diversity. The results of B.R. Martins *et al.* (2024) also confirmed that an increase in

microbiome diversity may be associated with improved agronomic characteristics of plants. Similar to their conclusions, the highest increase in diversity indices was observed in the combined treatment with preparations M1+M2. The conclusions of P. Gao *et al.* (2025) on the influence of environmental factors on the microbiome supported the data obtained in this study on the adaptation of the microbiome to changes in the environment, in particular after treatment with microbial preparations.

Comparison of the structure of potato rhizosphere microbiomes between variants based on taxonomic distance matrices showed pronounced differentiation of microbial communities under conditions of microbial preparation application. On the ordination diagram, the first axis summarised 38.2% of the variation in species composition, and the second axis summarised 19.4%. The centroids of the control variant samples were shifted to a separate area of the plane, while the variants with microbial preparations were grouped in other sectors. Samples M1 and M2 were located at a moderate distance from the control, demonstrating partial preservation of the microbiome structure with a simultaneous change in the relative contributions of individual taxa. For variants M3 and M1+M2, a more pronounced departure from the control centroids along the first axis was observed, reflecting a greater degree of taxonomic restructuring of the community. The study by B.R. Martins *et al.* (2024) confirms that different options for treating potatoes with microbial preparations can significantly alter the rhizosphere microbiome, which is reflected in its structure and richness. According to their results, variants treated with microbes often show an increase in species richness and a change in the ratio of taxa, which was also observed in this study, particularly in variants M1, M3 and M1+M2.

Analysis of the cluster structure based on distance matrices identified two main clusters of rhizosphere microbiomes. The first cluster was formed by the control variant and variant M2, for which the average pairwise distance between samples remained minimal. The second cluster combined variants M1, M3 and M1+M2, within which a subcluster with M3 and M1+M2 with lower intragroup dispersion was additionally identified. The average distance between the control variant and the M1+M2 variant was the maximum among all comparisons, indicating the greatest difference in the structure of the microbial community under combined treatment conditions. For variants M1 and M2, the values of intergroup distances relative to the control were lower than for M3 and M1+M2, which corresponded to the intermediate nature of structural changes. The results of the study of β-diversity of the potato rhizosphere microbiome confirm the conclusions of R.W.P.M. Rajapaksha *et al.* (2024), which showed that the microbiomes of different parts of the plant can vary significantly in structure and diversity depending on agronomic conditions. In this study, the variants with microbial preparations demonstrate a pronounced structural differentiation of microbial communities, reflecting the redistribution of dominant and subdominant taxa in response to treatment. Similar to a study by Z. Wang *et al.* (2021), which determined significant changes in the composition of the rhizosphere microbiome under the influence of microbial consortia, this study also observed that combined treatments M1+M2 led to the greatest changes in the structure of the microbial community. Z. Wang *et al.* (2021) found that increased diversity was associated with improved metabolic processes, which was also reflected in the increase in taxonomic restructuring in this study in variants with microbial preparations.

The structural differentiation of rhizosphere microbiomes in the experimental variants was interpreted as a result of the redistribution of dominant and subdominant taxa in response to the action of microbial preparations. The inclusion of M3 and especially the combination of M1+M2 was accompanied by the formation of configurations that differed from the control not only in species richness but also in the ratio of taxa that contributed most to intergroup diversity. The identified clusters reflected the presence of variants with a preserved microbial community structure (control, M2) and variants with a reformed community (M1, M3, M1+M2), which provided a basis for further analysis of the relationship between β-diversity, the functional profile of the metagenome, and the agrobiological parameters of plants.

Taxonomic profile of rhizosphere microorganisms under the influence of microbial preparations

Taxonomic analysis at the type level showed that

representatives of Proteobacteria, Actinobacteriota, Bacteroidota, and Firmicutes dominated in all variants of the experiment. In the control variant, the proportion of Proteobacteria was 43.1%, Actinobacteriota – 22.4%, Bacteroidota – 9.5%, Firmicutes – 7.3%, while the total contribution of other types to the metagenome structure was 17.7%. Under the conditions of treatment with microbial preparations, an increase in the relative abundance of Proteobacteria to 45.6-49.2% was observed in variants M1, M2, M3 and M1+M2. The maximum value of this indicator was recorded in the combined treatment variant M1+M2 (49.2%), accompanied by a simultaneous decrease in the proportion of "other" types to 12.5%. The proportion of Actinobacteriota in the experimental variants ranged from 20.1 to 23.8%, with slightly higher values in variant M2 (23.8%) compared to the control. For Bacteroidota, an increase in the proportion was noted in the variants with preparations M1 and M3: from 9.5% in the control to 10.5% in M1 and 11.7% in M3. In variants M2 and M1+M2, the proportion of Bacteroidota was at the level of 9.5-9.8%, close to the control values. The contribution of Firmicutes under the action of the preparations changed less significantly: in the control, it was 7.3%, in the M1 variant, 9.0%, in the M2, M3 and M1+M2 variants, 7.3-7.6%. Thus, the use of microbial preparations was accompanied by a shift in the taxonomic profile towards an increase in the proportion of Proteobacteria and, in some variants, Bacteroidota, while maintaining comparable proportions of Actinobacteriota and Firmicutes. Dominant types retained their leading role in the structure of the microbial community, and changes in their relative abundance determined the main differences between the control and experimental variants. The results of the taxonomic profile of potato rhizosphere microorganisms correlate with the study by F. Rasche *et al.* (2006), demonstrating that the use of transgenic potatoes with antibacterial properties changes the composition of the rhizosphere microbiome, in particular, increasing the proportion of beneficial bacteria such as *Pseudomonas* and *Bacillus*. Similar to their study, in this work, the use of microbial preparations led to an increase in the proportion of these genera in the structure of the microbiome, indicating the activation of components that promote plant growth.

At the family and genus level, a group of taxa associated with plant growth and nutrition was identified, including representatives of Plant Growth Promoting Rhizobacteria (PGPR), nitrogen fixers, phosphate-mobilising microorganisms, and phytohormone producers. The core group included the genera *Pseudomonas*, *Bacillus*, *Streptomyces*, *Flavobacterium*, *Rhizobium*, and *Sphingomonas*, which were present in most samples in all variants. In the control variant, the total relative abundance of these genera was 41.2%: *Pseudomonas* – 12.3%, *Bacillus* – 8.1%, *Streptomyces* – 6.2%, *Flavobacterium* – 4.0%, *Rhizobium* – 3.5%, *Sphingomonas* – 2.9%. In variant M1, there was an increase in the proportion of *Pseudomonas* to 14.5% and *Bacillus* to 9.8%, while the proportion of *Streptomyces* remained at 6.5%. The total contribution of these genera to the microbiome structure reached 44.9%, reflecting the increased representation of the PGPR component compared to the control. In variant M2, the growth was mainly concerned Actinobacteriota: the proportion of *Streptomyces* increased to 7.1%, while *Pseudomonas* and *Bacillus* remained at 13.0% and 8.5%, respectively. In variant M3, an increase in the relative abundance of *Flavobacterium* to 5.2% and *Rhizobium* to 4.3% was combined with an increase in the proportion of Pseudomonas to 15.0%, reflecting an increase in the participation of both PGPR and nitrogen-fixing components in the microbiome structure. In the combined treatment option M1+M2, the largest total contribution was recorded for the genera *Pseudomonas*, *Bacillus* and *Streptomyces*, which amounted to 30.8% (15.7%, 9.9% and 5.2%, respectively). In addition, this variant showed increased relative abundance of *Rhizobium* (4.0%) and *Sphingomonas* (3.0%). The total share of core genera in the M1+M2 variant reached 47.8%, which exceeded the control values (Fig. 2). A.C.F. Dias *et al.* (2013) emphasised that genetically modified potatoes contribute to changes in the composition of the rhizosphere microbiome compared to traditional varieties, which is also reflected in this study. In the combined treatment M1+M2, the largest increase in the proportion of the genera *Pseudomonas*, *Bacillus*, and *Streptomyces* was observed, indicating changes in the composition of rhizosphere bacteria associated with plant growth and development.

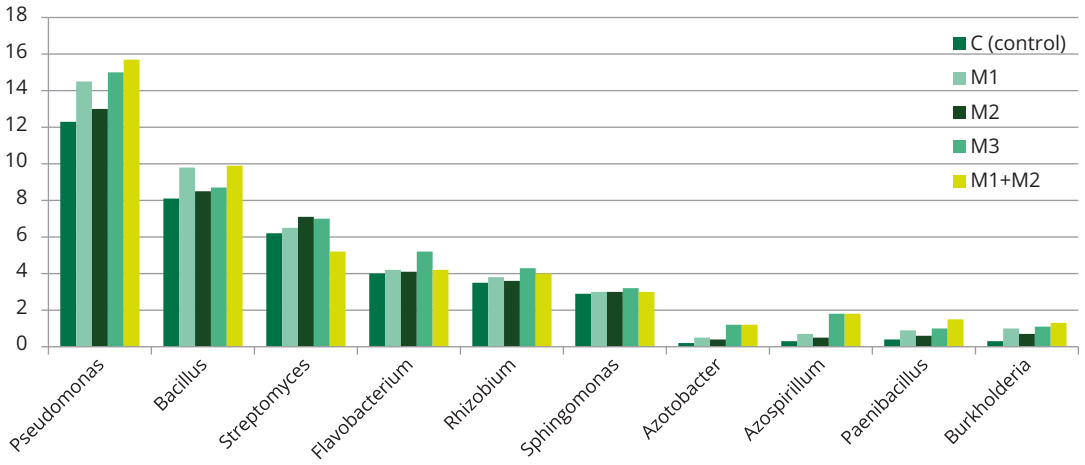

Figure 2. Relative abundance of key genera associated with potato growth, % of total bacterial community

Source: compiled by the authors based on research

A separate group consisted of genera that were absent or had a share of less than 0.5% in the control but reached noticeable values in the experimental variants. These included *Azotobacter*, *Azospirillum*, *Paenibacillus*, and *Burkholderia*. In variant M3, the proportion of *Azospirillum* reached 1.8% and *Azotobacter* 1.2%, while in the control, these genera were recorded at <0.5%. In variant M1+M2, the proportion of *Paenibacillus* was 1.5%, and *Burkholderia* was 1.3%, accompanied by an increase in the total proportion of nitrogen-fixing and phosphate-mobilising microorganisms. These changes indicated a reformatting of the functionally significant part of the microbial community due to the involvement of additional PGPR components and previously underrepresented taxa. A comparative analysis showed that under the conditions of treatment with microbial preparations, not only the ratio of dominant types changed, but also the structure of key families and genera associated with providing plants with nutrients and regulating growth processes. The increase in the proportion of *Pseudomonas*, *Bacillus*, *Streptomyces*, as well as the appearance or intensification of *Azotobacter*, *Azospirillum*, *Paenibacillus* and *Burkholderia* formed distinct taxonomic profiles of the experimental variants compared to the control and created prerequisites for further analysis of the functional potential of the potato rhizosphere metagenome. A. Boke (2024) also confirmed that the proportion of microorganisms that can suppress pathogens, in particular the genera *Burkholderia* and *Azotobacter*, increases in the rhizosphere of potatoes, which was found in this study after the combined use of microbial preparations. The observed increase in the relative abundance of these genera in the M1+M2 variant indicates the positive effect of microbial preparations on the formation of a healthy microbial community, which can potentially improve plant resistance to diseases.

Changes in the functional potential of the metagenome of rhizosphere microorganisms

Functional annotation of metagenomic sequences revealed the presence of a wide range of genes involved in providing plants with mineral nutrients. In all variants, genes for nitrogen fixation (nifH, nifD, nifK), phosphorus mobilisation and transport (phoD, pstS, pstA), potassium transport (kdpA, kdpB) and sulphur assimilation (cysD, cysN, cysK) were identified. The total relative share of these functional categories was 6.8% of the total number of functionally annotated genes in the control and 8.1-10.4% in the variants with microbial preparations. In the M1+M2 variant, this indicator reached 10.4%, which was the maximum value among all variants. Figure 3 shows the contribution of individual functional categories to this total indicator (the proportion of each category separately).

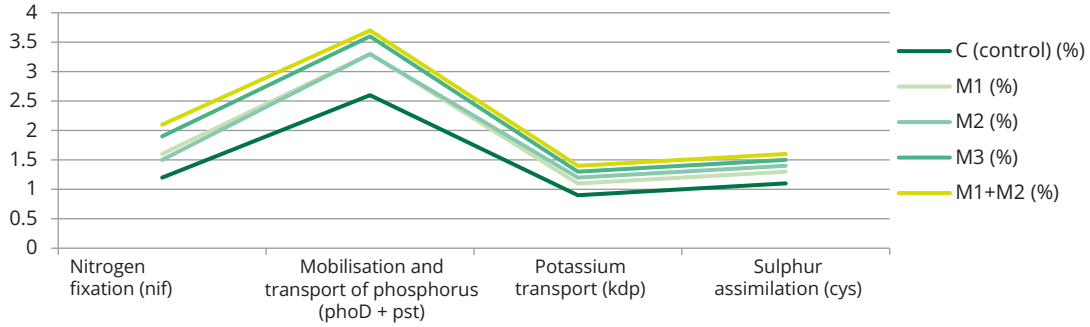

Figure 3. Relative proportion of individual functional categories of genes associated with plant nutrition, % of the total number of functionally annotated genes
Note: each column reflects the contribution of a separate category; the 0-4% scale is presented in percentages
Source: compiled by the authors based on research

Nitrogen fixation genes (nif complex) in the control variant accounted for 1.2% of the total number of functional annotations, while in variants M1, M2 and M3 their share was 1.6, 1.5 and 1.9%, respectively. In variant M1+M2, the relative proportion of nif genes reached 2.1%. The number of nifH annotations per 10^7 genera was 310 in the control, 410-430 in variants M1 and M2, 480 in variant M3, and 520 in variant M1+M2. This reflected an increase in the potential for biological nitrogen fixation under conditions of microbial preparation application, especially in the combined variant. For phosphorus-binding and phosphate-mobilising genes, the proportion of phoD and other organic phosphorus markers in the control was 1.5%, while in variants M1, M2 and M3 it was 1.9, 1.8 and 2.0%, respectively, and in variant M1+M2 it was 2.2%. The relative number of genes of highly related phosphate transport systems (pstS, pstA) increased from 1.1% in the control to 1.4-1.6% in the variants with preparations, with a maximum of 1.6% in variant M3. For potassium transport systems (kdpA, kdpB), the proportion of corresponding genes in the control was 0.9%, in variants M1, M2, M3 – 1.1-1.3%, and in M1+M2 – 1.4%. The total proportion of genes associated with sulphur assimilation (cysD, cysN, cysK) ranged from 1.1% in the control to 1.5% in M3 and 1.6% in the M1+M2 variant. The results of functional annotation of the potato rhizosphere metagenome confirm the conclusions of T.T. Alawiye & O.O. Babalola (2021), noting that sunflower rhizosphere microbiomes contain various functional gene groups that provide plants with essential nutrients. The study observed an increase in the relative number of genes involved in nitrogen fixation, phosphorus mobilisation, and potassium transport in variants with microbial preparations, indicating an expansion of the functional potential of the potato rhizosphere microbiome.

A comparison of the control and experimental variants showed that the use of microbial preparations was accompanied by an increase in the relative number of genes responsible for biological nitrogen fixation, mobilisation of mineral and organic phosphorus, and transport of potassium and sulphur. The highest values for most functional categories were recorded in variants M3 and M1+M2, which combined increased content of nif-, pho- and cys-genes. This reflected the expansion of the potential of rhizosphere microbial communities to provide plants with essential nutrients by changing the functional profile of the metagenome under the influence of microbial preparations.

Analysis of functional annotations related to stress resistance and phytoprotection revealed the presence of genes responsible for the synthesis of antibiotic-like compounds, siderophores, antioxidant enzymes, and systemic resistance inducers in the potato rhizosphere metagenome. In all variants, genes encoding enzymes for the biosynthesis of several non-specific polyketides and non-peptide polyketide synthases (NRPS/PKS-type clusters) were identified, as well as markers for the biosynthesis of phenazino-like compounds and lipopeptides. The total proportion of genes of potential phytoprotection was 4.5% of the total number of functional annotations in the control and 5.4-7.2% in variants with microbial preparations.

In the control variant, the proportion of genes associated with the biosynthesis of antibiotic-like compounds (in particular, individual NRPS/PKS clusters) was 1.8%, in variants M1, M2 and M3 – 2.2%, 2.1% and 2.6%, respectively, and in variant M1+M2 – 2.8%. For siderophore biosynthesis markers (including enterobactin and pyroverdin-type systems), the proportion of functional annotations increased from 1.2% in the control to 1.5-1.7% in variants M1 and M2 and to 1.9-2.0% in variants M3 and M1+M2. Antioxidant enzyme genes (catalase, superoxide dismutase, glutathione peroxidase) accounted for 1.1% in the control, while in the variants with microbial preparations, 1.3-1.6%, with maximum values in M3 and M1+M2 (1.6%) (Fig. 4).

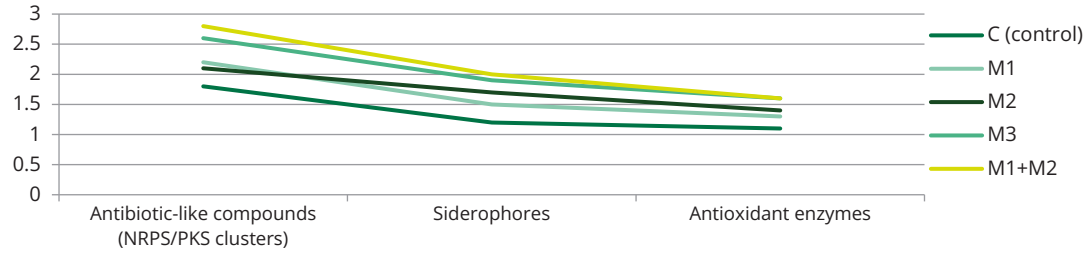

Figure 4. Relative abundance of functional groups of stress resistance and phytoprotection genes, % of the total number of functionally annotated genes
Source: compiled by the authors based on research

Genes associated with the induction of systemic resistance and regulation of plant stress responses were also identified, genes encoding 1-aminocyclopropane-1-carboxylate deaminase (acdS) activity and markers of the synthesis of compounds involved in the regulation of signalling pathways (in particular, phenolic metabolites). In the control, the relative proportion of such genes was 0.4%, while in variants M1, M3, and M1+M2, it was 0.6, 0.7, and 0.8%, respectively. In variant M2, the value remained at 0.5%, close to the control. Similar to R. Molefe *et al.* (2021), who found an increase in the proportion of functional genes in corn rhizosphere microbiomes after treatment, this study also noted an increase in functional genes associated with phytoprotection, in particular the biosynthesis of antibiotic-like compounds and siderophores. The largest increase in the proportion of these genes was recorded in variants M3 and M1+M2, indicating a shift in the functional profile of the microbiome in favour of components that help plants withstand biotic and abiotic stresses.

A comparison of functional markers of stress resistance between the variants showed that the use of microbial preparations caused an increase in the relative number of genes associated with the production of siderophores, antibiotic-like compounds and antioxidant enzymes, as well as an increase in the representation of genes potentially involved in the induction of systemic plant resistance. The most pronounced increase in the total share of these functional categories was observed in variants M3 and M1+M2. Within the scope of this study, this configuration of the functional profile of the rhizosphere metagenome was interpreted as an increased potential of microbial communities to create conditions that reduce the risk of biotic and abiotic stresses for potato plants.

The relationship between structural and functional changes in the microbiome and potato growth and productivity

A comparison of morphological and productive indicators showed that the variants with microbial preparations differed from the control both in terms of plant growth parameters and tuber yield structure (Table 3). The control variant recorded the lowest values for plant height (48.2 cm), tuber weight per plant (420 g) and number of tubers (7.8), as well as an increased proportion of small tubers (28.5%) with a relatively low proportion of large tubers (20.2%), which corresponds to the typical background without inoculation. In variant M1 (*Azotobacter* spp.), the indicators increased to 51.0 cm, 455 g, and 8.4 pcs., and the

proportion of small tubers decreased to 24.7% with an increase in large tubers to 21.5%, which is consistent with the effect of improved nitrogen supply and growth stimulation. In variant M2 (*Bacillus* spp., *Pseudomonas* spp.), the values increased to 52.6 cm, 470 g and 8.7 pcs., and the fractional structure shifted towards medium/large (54.5% and 22.0%) with a decrease in small tubers to 23.5%, which may reflect increased phosphorus nutrition and tuber filling. In variant M3 (complex consortium), some of the highest indicators were observed: 54.3 cm, 495 g and 9.1 pcs., with a minimum proportion of small tubers of 18.5% and an increase in medium/large tubers to 57.5% and 24.0%, which corresponds to the complex effect on nutrition and physiological stability of plants. In the combined M1+M2, the maximum values of height (55.1 cm), weight (510 g) and number of tubers (9.3 pcs.) were combined with the highest proportion of large fraction (25.1%) at 23.4% small, which is consistent with the complementarity of nitrogen and phosphorus supply. In general, in the variants with the preparations, the weight of tubers per plant increased from 420 g (control) to 455-510 g, and the number of tubers increased from 7.8 to 8.4-9.3 pieces, with a tendency to increase the proportion of large tubers to 21.5-25.1% compared to 20.2% in the control.

Table 3. Morphological and productive indicators of potatoes by variants

Variant	Plant height, cm (mean ± SD)	Tuber weight per plant, g (mean ± SD)	Number of tubers, pcs/plant (mean ± SD)	Percentage of tubers by fraction, % (small/medium/large)
C (control)	48.2 ± 1.5	420 ± 25	7.8 ± 0.5	28.5 / 51.3 / 20.2
M1	51.0 ± 1.6	455 ± 27	8.4 ± 0.6	24.7 / 53.8 / 21.5
M2	52.6 ± 1.7	470 ± 28	8.7 ± 0.6	23.5 / 54.5 / 22.0
M3	54.3 ± 1.8	495 ± 30	9.1 ± 0.6	18.5 / 57.5 / 24.0
M1+M2	55.1 ± 1.8	510 ± 32	9.3 ± 0.6	23.4 / 51.5 / 25.1

Source: compiled by the authors based on research

Analysis of the relationship between α-diversity indices of the rhizosphere microbiome and potato yield characteristics revealed positive correlations between the Shannon index and tuber weight per plant (r = 0.62) and the proportion of medium and large fractions (r = 0.58-0.65). These correlations can be explained by an increase in the proportion of functionally significant PGPR groups (in particular, nitrogen-fixing and phytohormone-producing bacteria, phosphate-mobilising taxa, as well as phytopathogen antagonists such as *Bacillus* spp. and *Pseudomonas* spp.), which enhance mineral nutrition and reduce stress on plants. In parallel, the increase in α-diversity could be accompanied by an increase in predicted functional gene categories (KEGG/KO) associated with nitrogen and phosphorus nutrition (nitrogen fixation, phosphorus mobilisation/transport, ion transport) and stress resistance mechanisms (synthesis of siderophores, antibiotic-like metabolites, antioxidant enzymes, induction of systemic resistance). Collectively, these factors could increase the availability of nutrients and stabilise the physiological state of plants during vegetation, which was manifested by an increase in tuber weight and an increase in the proportion of medium and large fractions. In variants with higher Shannon and Simpson indices (M3, M1+M2), higher average tuber weight and number of tubers per plant were also recorded. For the Chao1 index, a moderate correlation was found between the number of tubers (r = 0.54), reflecting the contribution of taxon richness to productivity. At the same time, no excessive variability in α-diversity was observed between variants, and the influence of intragroup diversity was manifested mainly through a combination of richness and evenness of the community. A comparison of β-diversity between variants, incorporating their productive characteristics, showed that the variants most distant from the control in terms of microbiome structure (M3, M1+M2) corresponded to variants with higher tuber weight and a greater contribution of medium and large fractions to the yield structure. A correlation (r = 0.59) was established for the average pairwise distance between the microbiome profiles and yield indicators, which indicated an association between the structural reorganisation of the rhizosphere community and an increase in

productive characteristics. Variants K and M2, which formed a common cluster on the ordination diagram, had similar values of yield and distribution of tubers by fractions.

A comparison of the relative abundance of key functional groups of microorganisms with the agrobiological characteristics of plants showed that an increase in the proportion of the genera *Pseudomonas*, *Bacillus*, *Rhizobium*, *Azotobacter*, *Azospirillum*, *Paenibacillus* and *Burkholderia* was associated with an increase in tuber weight per plant (r = 0.60-0.72 for the total proportion of PGPR genera) and a decrease in the proportion of small-sized tubers (r = -0.55). For the total proportion of nitrogen-fixing genes (nif) and genes associated with phosphorus mobilisation (phoD + pst), correlations with tuber weight were recorded (r = 0.63 and r = 0.66, respectively), while the proportion of potassium transport genes (kdp) and sulphur assimilation genes (cys) correlated mainly with tuber number and the proportion of the medium fraction (r = 0.48-0.57) (Fig. 5).

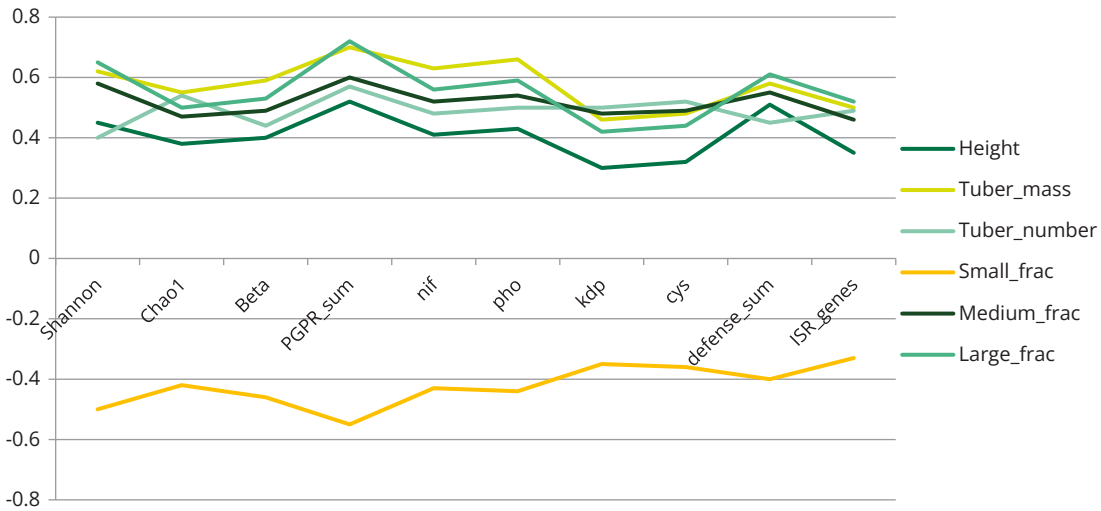

Figure 5. Correlation coefficients between microbiome indicators and potato yield
Source: compiled by the authors based on research

In the group of stress resistance and phytoprotection genes, the total relative proportion of markers for the biosynthesis of antibiotic-like compounds, siderophores, and antioxidant enzymes showed positive correlations with plant height (r = 0.51), tuber weight (r = 0.58) and the proportion of medium and large fractions (r = 0.55-0.61). The relative number of genes associated with the induction of systemic resistance (including acdS) correlated with the number of tubers per plant (r = 0.49) and the proportion of the large fraction (r = 0.52). This reflected the participation of functional components of the metagenome not only in providing nutrients, but also in creating conditions conducive to stable growth under the influence of potential stress factors. The results of the study of the functional potential of the potato rhizosphere metagenome are consistent with the conclusions of T. Zhao *et al.* (2024), noting that the interaction between the microbiome and the potato plant contributes to increased yield by improving plant nutrition and stress resistance. In this study, an increase in the proportion of genes responsible for biological nitrogen fixation and phosphorus, potassium and sulphur mobilisation was observed in variants with microbial preparations, which probably contributed to improved potato growth and productivity. M. Hemkemeyer *et al.* (2024) also emphasised the importance of the microbiome for improving the quality and quantity of potato yields, which is confirmed by the results of this study, where variants with microbial preparations, in particular M1+M2, showed a significant increase in tuber weight and the proportion of large fractions. According to the conclusions,

changes in the composition of the microbiome may be associated with better adaptation of plants to different agronomic conditions, which is reflected in the changes in the structure of the microbiome found in this study. A summary of the results showed that variants M3 and M1+M2 were characterised by a combination of increased α-diversity, pronounced structural differentiation of the microbiome relative to the control, an increased proportion of PGPR genera and functional genes associated with nutrition and stress resistance, with increased potato growth and productivity. Within the study, these variants were considered as configurations in which an optimal combination of parameters, "metagenome structure – functional potential – productivity", was formed, reflecting the consistency of taxonomic and functional changes in the rhizosphere microbiome with the agrobiological response of plants.

Analysis of the taxonomic structure of the rhizosphere metagenome identified a group of taxa consistently associated with the action of individual microbial preparations. For variant M1, a systematic increase in the proportion of the genera *Pseudomonas* and *Bacillus* was recorded, for variant M2 – an increased relative abundance of *Streptomyces*, and for variant M3 – a combination of an increased proportion of *Flavobacterium* and *Rhizobium*. In variant M1+M2, a complex of bioindicator genera was formed, which, in addition to *Pseudomonas* and *Bacillus*, included *Paenibacillus* and *Burkholderia*. *Azotobacter* and *Azospirillum* were found to be markers mainly for variant M3. The combination of these taxa formed characteristic "signatures" of the microbiome, according to which rhizosphere samples could be assigned to the corresponding treatment variant. At the level of functional potential, specific groups of genes characteristic of individual variants were identified. For M3, the maximum representation of nif genes and siderophore clusters was recorded, which was consistent with the increased proportion of *Rhizobium*, *Azotobacter* and *Azospirillum*. Variant M1 was characterised by enrichment of the metagenome with phosphorus mobilisation genes (phoD, pst) and individual NRPS/PKS clusters, while M2 was characterised by an increase in genes involved in the metabolism of organic forms of sulphur and actinobacterial clusters of secondary metabolism. The most comprehensive set of functional biomarkers was characteristic of the M1+M2 variant, where the total proportion of nitrogen fixation genes, phosphate mobilisation, potassium and sulphur metabolism, as well as genes associated with the biosynthesis of antibiotic-like compounds, siderophores, antioxidant enzymes and the induction of systemic resistance. The results of the functional analysis of the potato rhizosphere metagenome are consistent with the study by E. Chica *et al.* (2019), which showed that the presence of specific taxa, such as *Pseudomonas* and *Bacillus*, is associated with improved plant growth and health. In the presented study, these genera also showed an increased proportion in variants with microbial preparations, confirming their role in improving the functional potential of the potato rhizosphere microbiome. A study by X. Zhao *et al.* (2021) highlighted the importance of integrated metagenome and metabolomics analysis for identifying microbes with high biotechnological potential. Similar to their findings, this study recorded the maximum growth of functional genes associated with nitrogen fixation, phosphate mobilisation, and antibiotic-like compound synthesis in variants M3 and M1+M2, which contributed to improved agronomic characteristics of potatoes.

To summarise the data, an integral indicator of the biotechnological potential of the rhizosphere metagenome was formed, combining the structural parameters of the microbiome (α- and β-diversity, the proportion of dominant and PGPR genera), functional markers (nutrition and stress resistance genes) and indicators of potato growth and productivity. The value of the integral index was 0.48 for the control, 0.61 and 0.64 for M1 and M2, 0.72 for M3 and 0.75 for the combined variant M1+M2. Thus, all variants with microbial preparations exceeded the control in terms of a set of structural, functional and agronomic characteristics, with M3 and M1+M2 occupying the highest positions in the ranking. In terms of biotechnology, PGPR genera *Pseudomonas*, *Bacillus*, *Rhizobium*, *Paenibacillus*, *Burkholderia*, *Azotobacter* and *Azospirillum*, consistent with an increased content of genes for nitrogen fixation, phosphate mobilisation, siderophore synthesis and antibiotic-like compounds, were considered key biomarkers suitable for the selection of promising microbial consortia. An integrated assessment confirmed that

variants M3 and M1+M2 provided the optimal combination of a favourable metagenome structure, high functional potential, and increased potato productivity, which identified them as the most promising schemes for the application of microbial preparations under the conditions of the experiment.

CONCLUSIONS

The study determined that the use of microbial preparations was accompanied by modification of the potato rhizosphere metagenome at the taxonomic and functional levels and was associated with increased growth and productivity indicators. At a comparable sequencing depth, 11.8 million sequencing reads (after basic quality filtering) were obtained in all variants, and 2,950 taxonomic units (OTUs/ASVs) were identified. In the variants with preparations, an increase in α-diversity was recorded: the Shannon index increased from 4.82 in the control to 5.05-5.18, and Chao1 – from 830 to 940-975, which reflected the enrichment of the microbiome mainly due to rare OTUs/ASVs. Analysis of intergroup diversity showed the separation of some variants from the control profile and a restructuring of community structure. The taxonomic profile shifted towards the enrichment of PGPR genera (*Bacillus*, *Pseudomonas*, *Azotobacter*, *Rhizobium/Azospirillum*) and the strengthening of the role of taxa that were underrepresented in the control. The probable mechanisms were phytohormone-like metabolites, bioavailability of N and P, and local suppression of phytopathogens through siderophores.

At the functional level, the total share of gene categories associated with plant nutrition increased from 6.8% in the control to 8.1-10.4% in the variants with preparations; the maximum was recorded in M1+M2 (10.4%). At the same time, categories related to stress resistance (siderophores, antibiotic-like metabolites, antioxidant enzymes, resistance induction) increased. The most pronounced agronomic effects were observed in M3 and M1+M2: plant height increased from 48.2 ± 1.5 cm in the control to 54.3 ± 1.8 cm and 55.1 ± 1.8 cm; tuber weight increased from 420 ± 25 g to 495 ± 30 g and 510 ± 32 g; the number of tubers increased from 7.8 ± 0.5 to 9.1 ± 0.6 and 9.3 ± 0.6 pcs. The proportion of the large fraction increased from 20.2% in the control to 24.0% (M3) and 25.1% (M1+M2), while the small fraction decreased to 18.5% in M3. Correlation analysis confirmed the association between microbiome diversity and productivity: the Shannon index correlated positively with tuber weight ($r = 0.62$) and the proportion of medium and large fractions ($r = 0.58$-0.65), and correlations for the total proportion of beneficial microorganisms and functional genes with yield indicators reached $r \approx 0.6$-0.7. The integral indicator of biotechnological potential increased from 0.48 in the control to 0.61-0.75 in the variants with preparations, with a maximum in M3 and M1+M2. The limitation was that the experiment was conducted within a single agroecological context without long-term observation and without incorporating the transcriptomic level. Further research should address long-term and multi-location trials and testing of selected consortia in production crops.

ACKNOWLEDGEMENTS

None.

FUNDING

None.

CONFLICT OF INTEREST

None.

REFERENCES

[1] Alawiye, T.T., & Babalola, O.O. (2021). Metagenomic insight into the community structure and functional genes in the sunflower rhizosphere microbiome. *Agriculture*, 11(2), article number 167. doi: 10.3390/agriculture11020167.

[2] Boke, A. (2024). Assessment of microbial antagonism in the rhizosphere and non-rhizosphere of potato (*Solanum tuberosum* L.) at Gadab Hassasa district, West Arsi zone, Oromia regional state, Ethiopia. *Afribary*. Retrieved from https://surl.li/eleitt.

[3] Chen, G., Wu, C., Wang, F., Lyu, H., Lu, Y., Yan, C., Chen, J., Deng, Y., & Ge, T. (2022). Microbial community changes in different underground compartments of potato affected yield and quality. *3 Biotech*, 12, article number 106. doi: 10.1007/s13205-022-03167-6.

[4] Chica, E., Buela, L., Valdez, A., Villena, P., Peña, D., & Yarzábal, L.A. (2019). Metagenomic survey of the bacterial communities in the rhizosphere of three Andean tuber crops. *Symbiosis*, 79, 141-150. doi: 10.1007/s13199-019-00631-5.

[5] Convention on Biological Diversity. (1992, June). Retrieved from https://zakon.rada.gov.ua/laws/show/995_030#Text.

[6] Dias, A.C.F., Dini-Andreote, F., Hannula, S.E., Dini Andreote, F., e Silva, M.D.C.P., Salles, J.F., de Boer, W., van Veen, J., & van Elsas, J.D. (2013). Different selective effects onrhizosphere bacteria exerted by genetically modified versus conventional potato lines. *PLOS One*, 8(7), article number e67948. doi: 10.1371/journal.pone.0067948.

[7] Gao, P., *et al*. (2025). Environmental factors drive the changes of bacterial structure and functional diversity in rhizosphere soil of *Hippophae rhamnoides* subsp. *sinensis* rousi in arid regions of Northwest China. *Microorganisms*, 13(8), article number 1860. doi: 10.3390/microorganisms13081860.

[8] Ha, J., Gao, Y., Zhang, R., Li, K., Zhang, Y., Niu, X., Chen, X., Luo, K., & Chen, Y. (2021). Diversity of the bacterial microbiome associated with the endosphere and rhizosphere of different cassava (*Manihot esculenta* crantz) genotypes. *Frontiers in Microbiology*, 12, article number 729022. doi: 10.3389/fmicb.2021.729022.

[9] Hemkemeyer, M., Schwalb, S.A., Berendonk, C., Geisen, S., Heinze, S., Joergensen, R.G., Li, R., Lövenich, P., Xiong, W., & Wichern, F. (2024). Potato yield and quality are linked to cover crop and soil microbiome, respectively. *Biology and Fertility of Soils*, 60(4), 525-545. doi: 10.1007/s00374-024-01813-0.

[10] Hou, Q., Wang, W., Yang, Y., Hu, J., Bian, C., Jin, L., Li, G., & Xiong, X. (2020). Rhizosphere microbial diversity and community dynamics during potato cultivation. *European Journal of Soil Biology*, 98, article number 103176. doi: 10.1016/j.ejsobi.2020.103176.

[11] Imam, N., Belda, I., García-Jiménez, B., Duehl, A.J., Doroghazi, J.R., Almonacid, D.E., Thomas, V.P., & Acedo, A. (2021). Local network properties of soil and rhizosphere microbial communities in potato plantations treated with a biological product are important predictors of crop yield. *Msphere*, 6(4), 10-1128. doi: 10.1128/msphere.00130-21.

[12] Kiige, J.K., Kavoo, A.M., Mwajita, M.R., Mogire, D., Ogada, S., Wekesa, T.B., & Kiirika, L.M. (2025). Metagenomic characterization of bacterial abundance and diversity in potato cyst nematode suppressive and conducive potato rhizosphere. *PLOS One*, 20(5), article number e0323382. doi: 10.1371/journal.pone.0323382.

[13] Kiroyants, M.O. (2024). *Formation of the rhizosphere microbial biome of spring barley under different fertilization systems in typical chornozem*. (PhD thesis, National University of Life and Environmental Sciences of Ukraine, Kyiv, Ukraine).

[14] Kovalenko, V., Serdiuk, P., Shevych, A., Serdiuk, O., & Zakorko, V. (2024). Reaction of potato varieties to treatment with nitrogen-fixing bacteria and biopreparations with mycorrhiza. *Scientific Horizons*, 27(11), 32-40. doi: 10.48077/scihor11.2024.32.

[15] Lu, P., *et al*. (2023). Metagenomic insights into the changes in the rhizosphere microbial community caused by the root-knot nematode *Meloidogyne incognita* in tobacco. *Environmental Research*, 216(4), article number 114848. doi: 10.1016/j.envres.2022.114848.

[16] Martins, B.R., Radl, V., Treder, K., Michałowska, D., Pritsch, K., & Schloter, M. (2024). The rhizosphere microbiome of 51 potato cultivars with diverse plant growth characteristics. *FEMS Microbiology Ecology*, 100(8), article number fiae088. doi: 10.1093/femsec/fiae088.

[17] Melnyk, A., & Kyryk, M. (2020). The biological preparations efficiency research for potato alternaria blight decrease in terms of Western Forest-Steppe of Ukraine. *Interdepartmental Thematic Scientific Collection of Plant Protection and Quarantine*, 66, 157-167. doi: 10.36495/1606-9773.2020.66.157-167.

[18] Molefe, R.R., Amoo, A.E., & Babalola, O.O. (2021). Metagenomic insights into the bacterial community structure and functional potentials in the rhizosphere soil of maize plants. *Journal of Plant Interactions*, 16(1), 258-269. doi: 10.1080/17429145.2021.1936228.

[19] Mousa, S., Magdy, M., Xiong, D., Nyaruabaa, R., Rizk, S.M., Yu, J., & Wei, H. (2022). Microbial profiling of potato-associated rhizosphere bacteria under bacteriophage therapy. *Antibiotics*, 11(8), article number 1117. doi: 10.3390/antibiotics11081117.

[20] Qin, J., Bian, C., Duan, S., Wang, W., Li, G., & Jin, L. (2022). Effects of different rotation cropping systems on potato yield, rhizosphere microbial community and soil biochemical properties. *Frontiers in Plant Science*, 13, article number 999730. doi: 10.3389/fpls.2022.999730.

[21] Rajapaksha, R.W.P.M., Attanayaka, D.P.S.T.G., Vivehananthan, K., & McNevin, D. (2024). Metagenomic analysis of endophytic bacteria in seed potato (*Solanum tuberosum*). *Open Life Sciences*, 19(1), article number 20220897. doi: 10.1515/biol-2022-0897.

[22] Rajguru, B., Shri, M., & Bhatt, V.D. (2024). Exploring microbial diversity in the rhizosphere: A comprehensive review of metagenomic approaches and their applications. *3 Biotech*, 14(10), article number 224. doi: 10.1007/s13205-024-04065-9.

[23] Rasche, F., Hödl, V., Poll, C., Kandeler, E., Gerzabek, M.H., Van Elsas, J.D., & Sessitsch, A. (2006). Rhizosphere bacteria affected by transgenic potatoes with antibacterial activities compared with the effects of soil, wild-type potatoes, vegetation stage and pathogen exposure. *FEMS Microbiology Ecology*, 56(2), 219-235. doi: 10.1111/j.1574-6941.2005.00027.x.

[24] Song, J., Kong, Z.Q., Zhang, D.D., Chen, J.Y., Dai, X.F., & Li, R. (2021). Rhizosphere microbiomes of potato cultivated under *Bacillus* subtilis treatment influence the quality of potato tubers. *International Journal of Molecular Sciences*, 22(21), article number 12065. doi: 10.3390/ijms222112065.

[25] Wang, M., Zheng, C., Niu, Z., Liu, Z., Yang, Q., Zhang, J., Shang Q.B., Zhu F., & Wang, X. (2025). Microbial community in the potato rhizosphere in the Bashang region. *Chinese Journal of Eco-Agriculture,* 33(10), 1948-1956. doi: 10.12357/cjea.20240196.

[26] Wang, Z., Li, Y., Zhao, Y., Zhuang, L., Yu, Y., Wang, M., Liu, J., & Wang, Q. (2021). A microbial consortium-based product promotes potato yield by recruiting rhizosphere bacteria involved in nitrogen and carbon metabolisms. *Microbial Biotechnology*, 14(5), 1961-1975. doi: 10.1111/1751-7915.13876.

[27] Zhao, T., *et al*. (2024). Unveiling potato cultivars with microbiome interactive traits for sustainable agricultural production. *Plant, Cell & Environment*. doi: 10.1111/pce.70019.

[28] Zhao, X., Lin, C., Yuan, W., Ruan, S., Qi, G., Wang, R., & Tan, J. (2021). *Intergrated metagenomics and metabolomics analysis discovers nematicidal microbes, enzymes and metabolites from the plant rhizosphere microbiota*. doi: 10.21203/rs.3.rs-187899/v3.

Біотехнологічні аспекти формування метагеному ризосферних мікроорганізмів картоплі під дією мікробних препаратів

Віталій Оверченко

Кандидат сільськогосподарських наук, доцент
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
https://orcid.org/0009-0009-5435-6815

Юлія Коломієць

Доктор сільськогосподарських наук, професор
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
https://orcid.org/0000-0002-1919-6336

Анотація. Метою дослідження було оцінити вплив мікробних препаратів на структурно-функціональні характеристики ризосферного метагеному картоплі та пов'язані з цим зміни росту і продуктивності рослин. У межах вегетаційного досліду було отримано повногеномні метагеномні профілі ризосфери картоплі за п'яти варіантів: без обробки, три окремі мікробні препарати та їхня комбінація. Було отримано 11,8 млн секвенувальних родів, що пройшли базову якісну фільтрацію; ідентифіковано 2 950 таксономічних одиниць, виконано таксономічну й функціональну анотацію, розраховано показники різноманіття та проведено кореляційний аналіз із морфологічними й врожайними показниками. Встановлено, що у всіх варіантах домінували представники типів Proteobacteria, Actinobacteriota, Bacteroidota та Firmicutes, сумарна частка яких перевищувала 80 % структури метагеному, тоді як застосування мікробних препаратів супроводжувалося підвищенням видового багатства і різноманіття: індекс Шеннона зростав з 4,82 у контролі до 5,12-5,18 у дослідних варіантах, а оцінка багатства за Chao1 – з 830 до 960-975 таксонів. Було виявлено зростання частки родів, асоційованих із ростом рослин, а також відносної кількості генів, пов'язаних з азотфіксацією, мобілізацією фосфору, транспортуванням калію, засвоєнням сірки та ознаками стресостійкості, з максимальними значеннями у варіантах з третім препаратом і його комбінацією з першим. Ці варіанти забезпечували підвищення маси бульб з рослини до 495-510 г і збільшення частки середньої та крупної фракцій врожаю, причому коефіцієнти кореляції між сумарною часткою корисних мікроорганізмів, функціональними генами і продуктивністю досягали 0,6-0,7. Отримані результати засвідчили, що цілеспрямована мікробна обробка здатна формувати ризосферний метагеном із підвищеним біотехнологічним потенціалом і може бути використана агрономами-практиками, розробниками мікробних препаратів, селекціонерами картоплі та фахівцями з інтегрованого управління родючістю ґрунтів для оптимізації мікробних консорціумів у технологіях вирощування картоплі

Ключові слова: варіант; кількість; тип; рід; азотфіксація; фосфор

Received: 10.07.2025 Revised: 22.10.2025 Accepted: 27.11.2025 Published: 26.12.2025

BIOLOGICAL SYSTEMS: Theory and Innovation

ISSN 2706-8382
e-ISSN 2706-8390

Journal homepage: https://bioscience.com.ua/en
Vol. 16, No. 4, 2025, 90-100

UDC 636.085.52: 631.563.6

DOI: 10.31548/biologiya/4.2025.90

Lactobacteria for fermentation of vegetable raw materials

Alla Lukianets

PhD Student, Junior Researcher
Institute of Food Resources of the National Academy of Agrarian Sciences
02002, 4A Yevgen Sverstyuk Str., Kyiv, Ukraine
https://orcid.org/0000-0002-2120-9909

Svitlana Danylenko*

Doctor of Technical Sciences, Professor
Institute of Food Resources of the National Academy of Agrarian Sciences
02002, 4A Yevgen Sverstyuk Str., Kyiv, Ukraine
https://orcid.org/0000-0003-4470-4643

Abstract. The research relevance is determined by the need to select bacterial cultures capable of ensuring a stable and intensive course of plant biomass fermentation. A substantial criterion for the effectiveness of such cultures is their natural ecological adaptation to the substrate, which determines metabolic activity, tolerance to chemical components, and the ability to maintain key quality parameters of preservation. The study aimed to evaluate the morphological, physiological-biochemical, and fermentation properties of three lactic acid bacteria species isolated from different types of plant raw materials, as well as to determine their ability to grow and utilise carbohydrates in alfalfa juice, sugar beet tops juice, and their mixtures supplemented with sweet corn juice. The study was based on species identification methods based on determination of the fermentation profile, assessment of morphology, evaluation of growth characteristics under different temperature regimes, determination of the level of reducing sugar utilisation, and analysis of the dynamics of changes in substrate acidity during fermentation. The study established that isolates from plant raw materials exhibit a high adaptive capacity, manifested in an increase in cell numbers and active carbohydrate utilisation. The most intensive growth of heterofermentative cultures was observed in alfalfa juice and in the alfalfa-corn mixture, where their counts reached 8.68-8.77 log CFU/mL. Sugar beet tops supported enhanced sugar utilisation and pronounced changes in acidity but were less favourable for certain species. The addition of sweet corn juice improved the fermentation properties of all strains. The practical significance of the study is determined by identification of promising bacterial isolates suitable for the development of inoculants aimed at improving the fermentation of plant biomass, in particular for silage and haylage preservation

Keywords: alfalfa juice; sugar beet tops juice; bacterial isolates; carbohydrate substrate; acidity; metabolic activity

Suggested Citation:

Lukianets, A., & Danylenko, S. (2025). Lactobacteria for fermentation of vegetable raw materials. *Biological Systems: Theory and Innovation*, 16(4), 90-100. doi: 10.31548/biologiya/4.2025.90.

*Corresponding author

INTRODUCTION

The research relevance is determined by the need to select bacterial cultures capable of ensuring stable and intensive fermentation of plant biomass based on natural ecological adaptation to the substrate. Analysis of morphological, physiological-biochemical, and fermentation properties of lactic acid bacteria isolated from raw plant materials is necessary for development of efficient bacterial inoculants for silage and haylage production. S. Cheas *et al*. (2025) and B. Sionek *et al*. (2023) noted that only lactic acid fermentation can ensure a rapid decrease in environmental pH to levels, unuitable for most undesirable microorganisms. Complex carbohydrates, proteins, and vitamins are preserved without significant losses under such conditions, improving quality of feed. Therefore, silage starter preparations should include lactic acid bacteria cultures in combination with other microorganisms and enzymatic components that improve lactic acid fermentation while suppressing development of putrefactive, butyric acid-producing, yeast, and mold microflora.

The main advantages of lactic acid fermentation during silage preservation are the key role in maintaining the nutritional value of feed and stabilising fermentation processes. A. Fabiszewska *et al*. (2025) and C.O. Okoye *et al*. (2023) proved that lactic acid, formed by fermentation, is a substantial metabolic precursor in animal nutrition and, at the same time, an effective natural preservative. Accumulation of lactic acid suppresses undesirable degradation processes in the ensiled mass, particularly proteolysis, thereby contributing to improved preservation of protein compounds. The criteria for selecting lactic acid bacteria for use in silage and haylage additives are based on their ability to grow rapidly and dominate over the spontaneous microflora, efficiently produce lactic acid, tolerate acidic conditions, and ferment a broad range of carbohydrates. M.F. Akhtar *et al*. (2025) demonstrated that a range of *Lactobacillus plantarum* strains meet these requirements, which accounts for their widespread use in biological preparations for silage production. At the same time, given the limited activity of lactobacilli at the initial stages of fermentation under relatively high pH values, starter cultures are usually formulated as multicomponent compositions with the inclusion of species active under less acidic conditions.

Lactobacillus buchneri is notable among bacterial cultures used in modern silage. The use of this species was recommended by Y. Jin *et al*. (2024) and X. Lai *et al*. (2025) due to increased aerobic stability of silage, manifested by reduced activity of yeasts and molds after the feed comes into contact with oxygen. This positive effect is determined by the formation of acetic acid, which exerts an inhibitory action on microorganisms responsible for silage heating. As a result, silage masses treated by *Lactobacillus buchneri* have lower yeast and mold counts, slower development of microflora after air exposure, and a stable temperature that does not exceed ambient temperature even under warm weather conditions

C. Aslan & A.G. Filik (2025) noted the high tolerance of *Lactobacillus brevis* to acidic conditions and the presence of plant-derived inhibitors, including phenolic compounds, which is relevant for the ensiling of legumes, sugar beet tops, and grass mixtures. Metabolic plasticity of *Lactobacillus brevis* ensures efficient utilisation of both hexoses and pentoses of plant origin, providing stable fermentation even when readily available sugars are limited. M.P. Sychevskyi (2016) proved that in silage practice, *L. brevis* can be used as part of multicomponent bacterial inoculants in combination with homofermentative species. Such formulations combine a rapid decrease in acidity at the initial stages of fermentation with improved aerobic stability of the finished silage, thereby promoting nutrient preservation and enhancing feed quality. The study aimed to isolate and identify lactic acid bacteria from silage and haylage, evaluate their physiological-biochemical and growth characteristics and determine their fermentation activity and adaptability to plant substrates.

MATERIALS AND METHODS

Isolation and identification of lactic acid bacteria. The study was conducted in the Department of Biotechnology of the Institute of Food Resources of the National Academy of Sciences in 2022-2024. Isolates were obtained from fermented green biomass; specifically, corn silage and rye haylage were prepared under laboratory conditions. Fresh green corn intended for silage was chopped to a particle size of 1-2 cm using a knife chopper. The prepared biomass was tightly packed into laboratory

polyethylene bags, from which air was removed by vacuuming, after which the bags were hermetically sealed using a heat sealer. The hermetically sealed samples were stored at 20-25°C for 120 days. To isolate lactic acid bacteria (LAB), 20 g samples were collected under aseptic conditions, homogenised in a porcelain mortar with sterile sand, and diluted in 180 cm^3 of sterile physiological saline. Pure cultures were obtained by plating serial dilutions using the pour plate method on MRS agar (De Man *et al.*, 1960). Incubation was conducted at 37°C for 48 hours.

Investigation of morphological, cultural, and physiological properties of the cultures. Pure cultures were maintained in MRS broth at a temperature of (37 ± 2)°C. Phenotypic identification of the strains was performed using generally accepted morphological and physiological-biochemical tests. The physiological-biochemical properties of the strains were assessed by following parameters: amylase, catalase, and oxidase activities; reduction of nitrates to nitrites; ability to produce ammonia from arginine; tolerance to NaCl (5, 6.5, and 10%); tolerance to alkaline reaction of the medium (pH 3.0, 6.5, and 8.0); and growth in MRS at temperatures of 15 and 45°C. Morphological homogeneity of the cultures was monitored by microscopy with Gram staining (Beveridge, 2001). Microscopic analysis was performed using a Motic (Fischer Bioblock) microscope equipped with a built-in TopView video camera at a magnification of ×1000.

The biochemical profiles of the strains were investigated following (De Vos *et al.*, 2011) and using API 50 CHL test systems (bioMérieux, France). Culture identification was performed using apiweb™ software based on the obtained analytical index of the biochemical profile; the results were also recorded, and species designation was confirmed using ABIS online (n.d.). Changes in the main growth parameters in plant substrates were assessed as follows: biomass increase by plating serial dilutions, pH by potentiometric measurement, and reducing sugars by the ferricyanide method, according to (Mateles, 1960). Four types of juices were used for fermentation studies:

1. Alfalfa juice (*Medicago sativa*) (D1) – obtained by mechanical pressing of fresh green biomass.

2. Sugar beet tops juice (leaf biomass of *Beta vulgaris*) (D2) – obtained by mechanical pressing of fresh green biomass.

3. Mixture of alfalfa juice and sweet corn juice at a ratio of 9:1 (v/v) (D3).

4. Mixture of sugar beet tops juice and sweet corn juice at a ratio of 9:1 (v/v) (D4).

The juices were prefiltered through gauze and sterilised by autoclaving at 121°C for 10 minutes (to eliminate background microflora and stabilise the substrate). A 10% inoculum of the tested lactic acid bacteria was added to the prepared juices. Incubation was conducted at 34°C for 14 hours. To confirm LAB viability and growth, control plating on MRS agar was performed.

Statistical analysis. All experiments were performed in three independent replicates. Data were presented as the mean ± standard deviation (SD). Statistical significance of differences between mean values was determined by Duncan's test at a significance level of $p < 0.05$. Experimental studies on plants, including the collection of plant material, were conducted following institutional and national guidelines. The standards of the Convention on Biological Diversity (1992) were maintained.

RESULTS AND DISCUSSION

Studies by S. Danylenko *et al.* (2023) indicated that most effective bacterial isolates capable of improving fermentation processes predominantly originate from the same type of plant raw material or feed for which they are intended to be applied. This is associated with a high level of ecological adaptability of microorganisms: isolates that naturally occur in a substrate are better adapted to chemical composition, buffering capacity, concentration of phenolic compounds, and the spectrum of available carbohydrates of origin. As a result, bacterial isolates exhibit higher growth rates, enhanced organic acid production, and more stable development during fermentation. The study proved that isolates obtained from a specific type of feed raw material are among the most promising candidates for development of highly effective bacterial inoculants, as they combine natural adaptation with stable functional activity under conditions that are as close as possible to those of agrotechnological practice. Within the framework of the study, silage and haylage samples were used as sources of natural populations of lactic acid

bacteria capable of ensuring an intensive fermentation of plant biomass. Analysis of the samples revealed the presence of heterogeneous microbial communities dominated by representatives typical of ensiled feeds, belonging to the genera *Lactiplantibacillus*, *Lentilactobacillus*, and *Levilactobacillus*.

To obtain pure isolates, colonies exhibiting morphological characteristics typical of lactic acid bacteria were selected. Identification of the isolated lactic acid bacteria was conducted on the species level using commercial API 50 CH / API 50 CHL system (bioMérieux) and online ABIS system, which ensured high reliability in determination of the species status of the isolates. The results were presented in Table 1 and Table 2. Table 1 demonstrates the morphological and physiological-biochemical properties of the three isolated strains. The sources of isolation of the studied lactic acid bacteria included corn silage and rye haylage. These bacteria are Gram-positive straight or slightly curved rods, existing in single or short chains, occasionally in clusters. Isolate 1 has a homofermentative type of lactic acid fermentation without gas production from glucose, whereas Isolates 2 and 3 are heterofermentative.

Table 1. Morphological and physiological characteristics of lactic acid bacteria

Characteristics	Isolate 1	Isolate 2	Isolate 3
Source of Isolation	Corn silage	Rye haylage	Corn silage
Morphology	Straight or slightly curved rods, 1-3.5 μm long, 0.5-1.0 μm wide; occurring singly or in short chains	Straight or slightly curved rods, 2.0-5.0 μm long, 0.8-1.5 μm wide; occurring singly or in short chains of 3-15 cells	Short or medium-thick rods, 1.0-5.0 μm long, 0.8-1.2 μm wide; occurring singly, in short chains, or small clusters
Gram staining	+	+	+
Gas production from glucose	–	+	+
Catalase	–	–	–
Motility	–	–	–
Oxidase	–	–	–
Nitrate reduction	+	–	–
Gelatin hydrolysis	–	–	–
Growth at temperature			
15°C	+	+	+
45°C	–	–	–
Growth at pH			
3.0	±	–	–
6.5	+	+	+
8.0	+	+	+
Growth with NaCl, %			
5.0	+	+	+
6.5	+	+	+
10	+	–	–

Notes: "+" – positive; "–" – negative; "±" – weakly positive
Source: developed by the authors

Absence of catalase and motility was noted among all isolates; Isolate 1 reduces nitrates to nitrites. All species grow at 15°C and stop at at 45°C. Isolate 1 can grow at pH 3.0, whereas the other two do not. All three species grow efficiently at pH 6.5 and 8.0. *L. plantarum* tolerates up to 10% NaCl.

Table 2. Carbohydrate fermentation characteristics of lactic acid bacteria strains

Carbohydrate fermentation	Isolate 1	Isolate 2	Isolate 3
D-arabinose	–	–	–
D-xylose	–	+	+
Galactose	+	±	–
Gluconate	±	+	+
Glucose	+	+	+
Glycerol	–	–	–
Inulin	–	–	–
Lactose	+	+	–
Maltose	+	+	+
Mannitol	+	+	–
Mannose	+	–	–
Melezitose	+	+	–
Melibiose	+	+	+
Rhamnose	–	–	–
Raffinose	±	–	±
Ribose	+	+	+
Sucrose	+	–	±
Sorbitol	+	–	–
Trehalose	+	–	–
Fructose	+	+	+
Cellobiose	+	–	–

Notes: "+" – positive; "–" – negative; "±" – weakly positive
Source: developed by the authors

Table 2 shows the ability of the three isolates to ferment various carbohydrates. The isolates differed significantly in their carbohydrate fermentation profiles, as Isolate 1 exhibited broadest profile, typical of *Lactiplantibacillus plantarum*; Isolate 2 showed a moderately broad profile, characteristic of *Lentilactobacillus buchneri*; whereas Isolate 3 displayed the narrowest fermentation spectrum, corresponding to the species traits of *Levilactobacillus brevis*. At the next stage of the study, the optimal cultivation temperature for the LAB strains was determined. The effect of cultivation temperature (25-40°C) on the cell counts of the three studied bacterial cultures is shown in Figure 1. The results are expressed as Log 10 CFU/mL for assessment of microbial growth intensity under different temperature regimes.

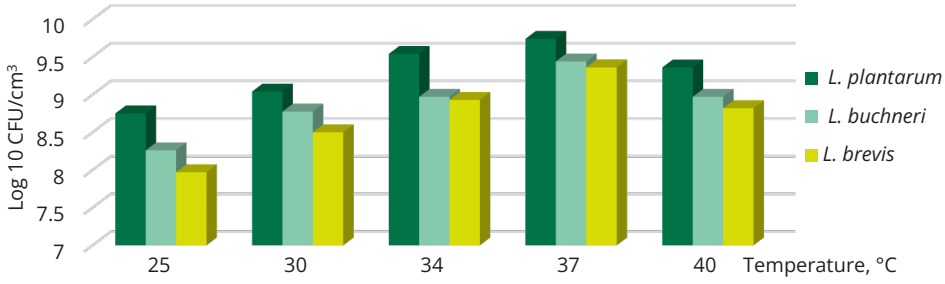

Figure 1. Effect of cultivation temperature on the cell counts of the studied lactic acid bacteria
Source: developed by the authors

Highest growth rates were observed at 34-37°C, correlating to optimal conditions for most mesophilic lactic acid bacteria. At 30°C, relatively high growth was noted, although lower than the

maximum, whereas 25°C provided the minimal growth among the tested temperature conditions. Increase in temperature to 40°C provided a moderate decrease in activity, which could be determined by heat-induced stress. All subsequent experiments were conducted at 34°C. Comparison of viable cell counts in juices D1-D4 demonstrates different levels of strain adaptation to the chemical composition of the substrates and the intensity of their growth (Fig. 2).

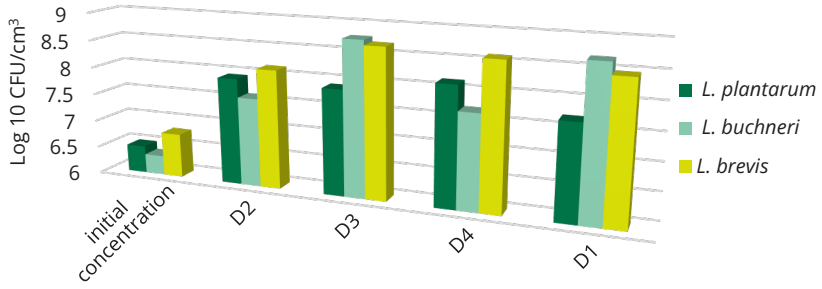

Figure 2. Cell counts of the studied lactic acid bacteria in different plant juices
Source: developed by the authors

The study established that juices D1 and D2 were the most favourable media for the growth of the heterofermentative lactic acid bacteria *Lentilactobacillus buchneri* and *Levilactobacillus brevis*. In these variants, cell counts reached 8.68-8.77 log CFU/mL, indicating a high level of adaptation and intensive metabolism of these species in protein-carbohydrate substrates. *Lactiplantibacillus plantarum* exhibited stable growth in all tested juices (7.7-8.14 log CFU/mL), although at slightly lower levels compared to the heterofermentative species, which may be related to the characteristics of the carbohydrate composition of the plant juices and the high buffering capacity of the substrates.

D2 Juice provided a less optimal enrivonment for *L. buchneri* (7.6-7.7 log CFU/mL), whereas *L. brevis* maintained high cell counts (8.14-8.60 log CFU/mL), indicating higher tolerance of this species to specific components of D2 juice. The addition of 10% sweet corn juice to alfalfa or sugar beet tops juice improved growth of all studied LAB, confirming the feasibility of carbohydrate-enriched mixtures for optimisation of the fermentation of plant raw materials. The obtained results indicate the potential of juices D1 and D2, either standalone or in combination with juices D3 and D4, as substrates for the cultivation and application of formulations based on *L. plantarum*, *L. buchneri*, and *L. brevis* in silage production and plant biomass biopreservation technologies. Figure 3 shows the changes in pH in various plant juices under the influence of the investigated bacterial cultures.

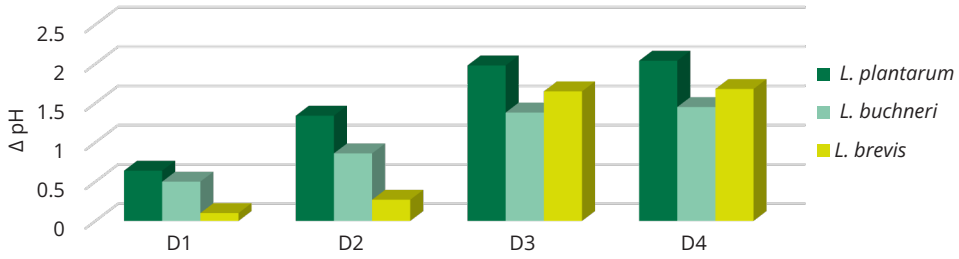

Figure 3. Changes in pH in different plant juices under the influence of the studied bacterial cultures
Source: developed by the authors

Following Figure 3, the ΔpH values demonstrate significant differences between substrates and LAB species. The smallest change in acidity was noted in D1 (ΔpH 0.10-0.64), indicating

low lactic acid fermentation intensity. In contrast, D2 juice demonstrated highest pH decrease (ΔpH 1.34-1.98), and the addition of 10% sweet corn juice further increased acidification (up to ΔpH 2.04 for *L. plantarum*). The homofermentative *L. plantarum* consistently demonstrated highest pH reduction capacity, whereas the heterofermentative *L. buchneri* produced the lowest ΔpH values, which corresponds to metabolic profile. *L. brevis* exhibited high activity in D2 and D4 but low activity in D1 juice. Figure 4 demonstrates initial concentration of sugar reduction in the plant juices, as well as the amounts of sugars consumed by individual lactic acid bacteria strains (*L. brevis*, *L. plantarum*, *L. buchneri*) during fermentation after 24 hours. The difference between the initial sugar levels in the juices and the values for each species reflects the metabolic activity of the strain, hence, ability to utilise available plant-derived hexoses and pentoses.

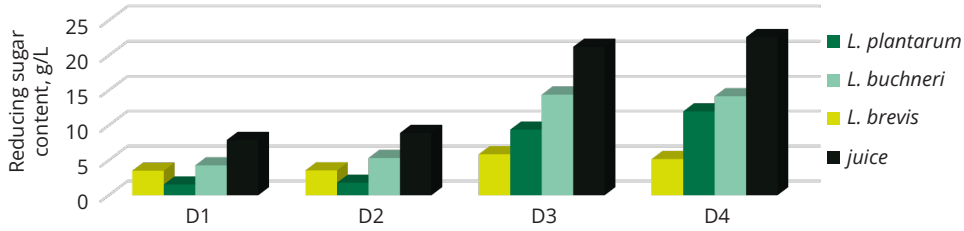

Figure 4. Consumption of reducing sugars by different strains in plant juices
Source: developed by the authors

Analysis of carbohydrate consumption by LAB strains in D1 juice demonstrated that *L. brevis* consumed 3.52-3.56 units, utilising approximately half of the available sugars. *L. plantarum* assimilated only 1.54-1.79 units, which indicates a limited amount of substrate for the homofermentative pathway. *L. buchneri* consumed the largest amount, 4.28-5.33 units, which corresponds to ability to convert residual sugars into acetic acid. D2 Juice improved initial concentration of reducing sugars threefold, demonstrating much more intensive course of lactic acid fermentation. Due to high initial sugar content of this juice, higher carbohydrate consumption was noted among all strains, namely *L. brevis*: 5.18-5.86 units, *L. plantarum*: 9.39-12.00 units, and the highest consumption by *L. buchneri*: 14.16-14.39 units. All strains utilised more than half of the available sugars, with *L. buchneri* consuming up to 65-67%, which is characteristic of heterofermentative metabolism under conditions of sufficient substrate.

The conducted studies demonstrated that lactic acid bacteria isolated from silage and haylage exhibit a high degree of adaptation to plant raw materials, manifested by stable growth, active carbohydrate utilisation, and intensive fermentation processes. The isolated strains, identified as *Lactiplantibacillus plantarum*, *Lentilactobacillus buchneri*, and *Levilactobacillus brevis*, differed in their metabolic profiles and type of lactic acid fermentation. The heterofermentative strains showed the highest cell counts in alfalfa juice and its mixtures with sweet corn juice, whereas *L. plantarum* produced the most pronounced pH reduction. Beet top juice promoted active consumption of reducing sugars and significant changes in acidity, especially for *L. buchneri*, and the addition of 10% sweet corn juice enhanced the growth and fermentation activity of all strains. The obtained results confirm the potential of ecologically adapted isolates for the development of effective bacterial formulations aimed at optimising silaging and bioconservation of plant biomass.

Sugar beet tops juice provides optimal conditions for development of studied lactobacilli. *L. buchneri* strain consistently consumed the largest amount of carbohydrates in all media, highlighting the key role in acetic acid formation in silage, while *L. plantarum* demonstrated highest adaptability in media with high hexose concentrations. The obtained results are consistent with the data of P. McDonald *et al*. (1991) on the influence of the chemical composition of plant substrates on the growth and fermentation activity of lactic acid bacteria. The high cell counts of *L. buchneri* and *L. brevis* during fermentation of juices D1 and

D3 can be attributed to the combination of available carbohydrates and a substantial content of nitrogenous compounds and mineral components, which create favourable conditions for heterofermentative metabolism. Similar trends were reported by N.A. Khan *et al*. (2016) for alfalfa silage and grass mixtures, where the addition of carbohydrate components (corn, cereals) improves LAB growth and fermentation stability.

High values of log CFU/mL for *L. buchneri* and *L. brevis* in the D3 juice mixture are consistent with reports S.J.W.H. Oude Elferink *et al*. (2001) and Z.G. Weinberg & Y. Chen (2013) that heterofermentative LAB, particularly *L. buchneri*, efficiently colonise substrates rich in fermentable sugars and can convert lactic acid into acetic acid and 1,2-propanediol, thereby improving aerobic stability of silage. Therefore, such juice mixtures can be used as model systems for selection of strains with high silage application potential. D2 juice proved to be a less favourable medium for *L. buchneri*, which may be related to specific features of its carbohydrate–mineral profile, the presence of phenolic compounds, or increased buffering capacity that inhibit the growth of certain LAB. At the same time, *L. brevis* was consistent in high performance across all variants, correlating with literature data by K. Suzuki *et al.* (2006) on ability to grow in a wide range of plant substrates, including brewing wort, fermented vegetables, and plant extracts. Such tolerance renders *L. brevis* a promising component of multistrain compositions for biopreservation. Compared to heterofermentative species, *L. plantarum* exhibited slightly lower cell counts, although growth remained high in all media (above 7.7 log CFU/mL). This is consistent with the concept of A.S. Oliveira *et al*. (2017) that *L. plantarum*, as a universal homofermentative species, can efficiently lower pH and provide effective preservation, but does not always reach maximum biomass under conditions of high buffering capacity and relatively low levels of easily fermentable sugars. J.M. Wilkinson & D.R. Davies (2013) noted that the combination of *L. plantarum* with *L. buchneri* and *L. brevis* can be used for both rapid acidification of the substrate and improved aerobic stability due to the production of acetic acid by heterofermentative strains.

A substantial practical aspect is the positive effect of sweet corn juice on alfalfa and sugar beet tops juices. Enrichment of the substrate with readily available carbohydrates improved cell counts of all studied LAB, confirming the feasibility of combined protein- and carbohydrate-rich feed components during ensiling, including crops with high buffering capacity (alfalfa, sugar beet tops). These results are consistent with reports by R.E. Muck *et al*. (2018) on efficiency of carbohydrate additives and bacterial inoculants in improvement of silage fermentation quality and reduction of dry matter losses. Overall, the obtained data confirm that selection of lactic acid bacteria strains should incorporate not only overall fermentative activity but also specific adaptation to particular plant substrates. Alfalfa and sugar beet tops juices, especially when combined with sweet corn juice, can be considered a promising media for the cultivation and application of LAB consortia aimed at improving the efficiency of ensiling and the bioconservation of plant biomass. The obtained results can be used for the development of effective inoculants for the fermentation of plant biomass, particularly for the ensiling of legumes and sugar beet residues.

CONCLUSIONS

As a result of the conducted studies, three lactic acid bacterial isolates were isolated and identified from corn silage and rye haylage, namely *Lactiplantibacillus plantarum*, *Lentilactobacillus buchneri*, and *Levilactobacillus brevis* species. These isolates differed in lactic acid fermentation type and carbohydrate fermentation profiles. Optimal cultivation temperature for all investigated strains, at which the maximum cell counts were observed, was 34-37°C whereas minimal growth was recorded at 25°C, and an increase in temperature to 40°C resulted in a moderate decrease in metabolic activity. Highest growth levels of the heterofermentative strains *L. buchneri* and *L. brevis* were noted to be in alfalfa juice and alfalfa-corn mixtures, with cell counts reaching 8.68-8.77 log CFU/mL, indicating a high level of adaptation to protein-carbohydrate substrates. The homofermentative strain *Lactiplantibacillus plantarum* demonstrated stable growth in all tested juices, with cell counts of 7.7-8.14 log CFU/mL, and highest lactic acid production capacity, resulting in a pH decrease of ΔpH 1.98-2.04 in carbohydrate-enriched media.

Beet top juice exhibited the highest initial concentration of reducing sugars and supported their intensive consumption by all strains; *L. buchneri* utilised 65-67% of the available carbohydrates, which was consistent with its heterofermentative metabolic profile. The addition of 10% corn juice to alfalfa or beet top juices promoted increased cell counts of all investigated LAB, intensified the consumption of reducing sugars, and enhanced medium acidification compared with single-component substrates. The obtained results can be used as a scientific basis for the development and production of domestic bacterial inoculants based on highly efficient lactic acid bacteria adapted to plant raw materials, which can optimise the silaging and bioconservation processes.

ACKNOWLEDGEMENTS

None.

FUNDING

None.

CONFLICT OF INTEREST

None.

REFERENCES

[1] ABIS online. (n.d.). Retrieved from https://www.tgw1916.net/ABIS/bacteria_abis.html.

[2] Akhtar, M.F., Wenqiong, C., Umar, M., & Changfa, W. (2025). Biochemical properties of lactic acid bacteria for efficient silage production: An update. *Frontiers in Microbiology*, 16, article number 1581430. doi: 10.3389/fmicb.2025.1581430.

[3] Aslan, C., & Filik, A.G. (2025). Effects of native *Lactobacillus brevis* (MF098783) strain on the fermentation profile, aerobic stability, and digestibility of wheat straw silage. *Turkish Journal of Agriculture – Food Science and Technology*, 13(9), 2784-2789. doi: 10.24925/turjaf.v13i9.2784-2789.8074.

[4] Beveridge, T. (2001). Use of the Gram stain in microbiology. *Biotechnic & Histochemistry*, 76(3), 111-118. doi: 10.1080/bih.76.3.111.118.

[5] Cheas, S., Suntara, C., & Cherdthong, A. (2025). Bioactive roles of lactic acid bacteria in enhancing corn silage quality and ruminant dietary fiber utilization: A comprehensive review. *Bioactive Carbohydrates and Dietary Fibre*, 34, article number 100512. doi: 10.1016/j.bcdf.2025.100512.

[6] Convention on Biological Diversity. (1992, June). Retrieved from https://zakon.rada.gov.ua/laws/show/995_030#Text.

[7] Danylenko, S., Lukianets, A., & Verbytskyi, S. (2023). Inoculant efficiency in corn silage preparation. *Bulletin of the Kyrgyz National Agrarian University*, 21(2), 62-71.

[8] De Man, J.C., Rogosa, M., & Sharpe, M.E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology*, 23(1), 130-135. doi: 10.1111/j.1365-2672.1960.tb00188.x.

[9] De Vos, P., Garrity, G.M., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., & Whitman, W.B. (Eds.). (2011). *Bergey's manual of systematic bacteriology: Vol. 3: The firmicutes*. New York: Springer.

[10] Fabiszewska, A., *et al*. (2025). Enhancing propionic acid formation and biogas yield from grass silage via co-fermentation of *Pediococcus acidilactici* and *Lentilactobacillus buchneri*. *Bioresource Technology*, 437, article number 133107. doi: 10.1016/j.biortech.2025.133107.

[11] Jin, Y., Wang, P., Li, F., Yu, M., Du, J., Zhao, T., Yi, Q., Tang, H., & Yuan, B. (2024). The effects of *Lactobacillus plantarum* and *Lactobacillus buchneri* on the fermentation quality, *in vitro* digestibility, and aerobic stability of *Silphium perfoliatum* L. Silage. *Animals*, 14(15), article number 2279. doi: 10.3390/ani14152279.

[12] Khan, N.A., Yu, P., Ali, M., Cone, J.W., & Hendriks, W.H. (2015). Nutritive value of maize silage in relation to dairy cow performance and milk quality. *Journal of the Science of Food and Agriculture*, 95(2), 238-252. doi: 10.1002/jsfa.6703.

[13] Lai, X., Wang, H., Peng, R., Chen, Z., Xiang, Y., & Yan, L. (2025). Different commercial microbial additives influence fermentation quality and microbial community of king grass silage. *Fermentation*, 11(5), article number 264. doi: 10.3390/fermentation11050264.

[14] Mateles, R.I. (1960). Ferricyanide reduction method for reducing sugars. *Nature*, 187, 241-242. doi: 10.1038/187241a0.
[15] McDonald, P., Henderson, A.R., & Heron, S.J.E. (1991). *The biochemistry of silage* (2nd ed.). Marlow: Chalcombe Publications.
[16] Muck, R.E., Nadeau, E.M.G., McAllister, T.A., Contreras-Govea, F.E., Santos, M.C., & Kung, L. (2018). Silage review: Recent advances and future uses of silage additives. *Journal of Dairy Science*, 101(5), 3980-4000. doi: 10.3168/jds.2017-13839.
[17] Okoye, C.O., Wang, Y., Gao, L., Wu, Y., Li, X., Sun, J., & Jiang, J. (2023). The performance of lactic acid bacteria in silage production: A review of modern biotechnology for silage improvement. *Microbiological Research*, 266, article number 127212. doi: 10.1016/j.micres.2022.127212.
[18] Oliveira, A.S., *et al*. (2017). Meta-analysis of effects of *Lactobacillus plantarum* inoculation on fermentation, aerobic stability, and nutritive value of silages. *Journal of Dairy Science*, 100(6), 4587-4603. doi: 10.3168/jds.2016-11815.
[19] Oude Elferink, S.J.W.H., Krooneman, J., Gottschal, J.C., Spoelstra, S.F., Faber, F., & Driehuis, F. (2001). Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri*. *Applied and Environmental Microbiology*, 67(1), 125-132. doi: 10.1128/AEM.67.1.125-132.2001.
[20] Sionek, B., Szydłowska, A., Küçükgöz, K., & Kołożyn-Krajewska, D. (2023). Traditional and new microorganisms in lactic acid fermentation of food. *Fermentation*, 9(12), article number 1019. doi: 10.3390/fermentation9121019.
[21] Suzuki, K., Iijima, K., Sakamoto, K., Sami, M., & Yamashita, H. (2006). A review of hop resistance in beer spoilage lactic acid bacteria. *Journal of the Institute of Brewing*, 112(2), 173-191. doi: 10.1002/j.2050-0416.2006.tb00247.x.
[22] Sychevskyi, M.P. (2016). The effectiveness of Senosil for silage preservation. *Grain Products and Mixed Fodder's*, 3(63), 16-21. doi: 10.15673/gpmf.v63i3.215.
[23] Weinberg, Z.G., & Chen, Y. (2013). Effects of storage period on the composition of whole crop wheat and corn silages. *Animal Feed Science and Technology*, 185(3-4), 196-200. doi: 10.1016/j.anifeedsci.2013.08.009.
[24] Wilkinson, J.M., & Davies, D.R. (2013). The aerobic stability of silage: Key findings and recent developments. *Grass and Forage Science*, 68(1), 1-19. doi: 10.1111/j.1365-2494.2012.00891.x.

Лактобактерії для ферментації рослинної сировини

Алла Лукянець

Аспірант, молодший науковий співробітник
Інститут продовольчих ресурсів НААН
02002, вул. Євгена Сверстюка, 4А, м. Київ, Україна
https://orcid.org/0000-0002-2120-9909

Світлана Даниленко

Доктор технічних наук, професор
Інститут продовольчих ресурсів НААН
02002, вул. Євгена Сверстюка, 4А, м. Київ, Україна
https://orcid.org/0000-0003-4470-4643

Анотація. Актуальність даного дослідження зумовлена необхідністю відбору бактеріальних культур, здатних забезпечувати стабільний та інтенсивний перебіг ферментації рослинної біомаси. Важливим критерієм ефективності таких культур є їх природна екологічна адаптація до субстрату, що визначає метаболічну активність, толерантність до хімічних компонентів та здатність підтримувати ключові показники якості консервування. Метою цього дослідження було оцінити морфологічні, фізіолого-біохімічні та ферментаційні властивості трьох видів молочнокислих бактерій, ізольованих із різних типів рослинної сировини, а також визначити їхню здатність до росту й утилізації вуглеводів у соку люцерни, соку гички цукрового буряка та їх сумішах із додаванням соку солодкої кукурудзи. У роботі застосовувалися методи видової ідентифікації, засновані на визначенні ферментаційного профілю, оцінці морфологічних ознак, дослідженні ростових характеристик за різних температурних режимів, встановленні рівня споживання відновлювальних цукрів та аналізі динаміки змін кислотності субстрату під час ферментації. Було встановлено, що ізоляти, отримані з рослинної сировини, характеризуються високою адаптивною здатністю, що проявлялося у збільшенні кількості клітин та активному використанні вуглеводів. Найінтенсивніший ріст гетероферментативних культур спостерігався у соку люцерни та в суміші люцерни з кукурудзою, де їх чисельність досягала 8,68-8,77 log КУО/мл. Гичка цукрового буряка сприяла посиленому споживанню цукрів і вираженим змінам кислотності, однак була менш сприятливою для окремих видів. Додавання соку солодкої кукурудзи покращувало ферментаційні властивості всіх досліджуваних штамів. Практичне значення роботи полягає у виявленні перспективних бактеріальних ізолятів, придатних для розроблення інокулянтів, спрямованих на підвищення ефективності ферментації рослинної біомаси, зокрема при консервуванні силосу та сінажу

Ключові слова: сік люцерни; сік гички цукрового буряка; бактеріальні ізоляти; вуглеводний субстрат; кислотність; метаболічна активність

Received: 21.06.2025 Revised: 25.10.2025 Accepted: 27.11.2025 Published: 26.12.2025

BIOLOGICAL SYSTEMS: Theory and Innovation

ISSN 2706-8382
e-ISSN 2706-8390

Journal homepage: https://bioscience.com.ua/en
Vol. 16, No. 4, 2025, 101-117

UDC 632:579.2

DOI: 10.31548/biologiya/4.2025.101

Bacteria of *Bacillus* genus as efficient and safe plant protection agent against pathogenic microorganisms

Sofia Kozlova*

Postgraduate Student, Junior Researcher
Ukrainian Institute for Plant Variety Examination
03041, 15 Horikhuvatskyi Shliakh Str., Kyiv, Ukraine
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
https://orcid.org/0009-0000-5532-6866

Abstract. The study aimed to establish correlation between antagonistic activity of *Bacillus* strains and their effect on plant growth and soil microbial balance. Experiment was based on combination of microbiological, physiological and statistical methods, including agar well method for determining antagonism, serial dilution method for counting colonies, triphenyl tetrazolium chloride method for assessing dehydrogenase activity, and analysis of variance (ANOVA) for result reliability verification. Experiment determined that *B. subtilis* has a higher inhibitory capacity than *B. amyloliquefaciens*, determining that average diameters of inhibition were 10-15% larger, while growth inhibition index was larger by 5-8%. Lesion index in *Fusarium oxysporum* dropped by 33.0 percentage points (p.p.) (from 53.8% to 20.8%), in *Alternaria solani* by 29.0 p.p., and in *Pseudomonas syringae* by 27.1 p.p. Proportion of healthy plants in control group improved from 51.5-56.3% to 86.2-87.3% with *B. subtilis* and 82.4-84.1% with *B. amyloliquefaciens*. Height, number of leaves and root mass improved by 20-40% compared to control, exceeding efficiency of biological standard by 5-10%, therefore demonstrating stimulative effect of biological products on plant growth. Experiment detected no phytotoxicity or soil degradation. Biological preparations ensured stable saprophyte (1.8×10^6 CFU/g), actinomycetes (1.9×10^6 CFU/g) and fungi (1.8×10^5 CFU/g) count, while dehydrogenase activity reached 103% compared to control. Results of experiment confirmed that *Bacillus subtilis* and *Bacillus amyloliquefaciens* are efficient antagonists of phytopathogens and safe biostimulants that can improve productivity and stabilise soil microbiocenosis. They are an efficient, environmentally sustainable alternative to chemical fungicides in sustainable farming systems

Keywords: antagonism; biocontrol activity; damage index; environmental safety; rhizosphere

INTRODUCTION

Research relevance is determined by growing need to identify environmentally safe alternatives to chemical plant protection products in modern agricultural production. Development of agriculture and excessive use of fungicides and pesticides cause soil degradation, loss of microbial diversity and formation of resistant strains of phytopathogens. In this regard, introduction of biological products based on microorganisms capable of not only inhibiting development of pathogens but also stimulating growth of cultivated plants, restoring natural balance of agroecosystems, is

Suggested Citation:

Kozlova, S. (2025). Bacteria of *Bacillus* genus as efficient and safe plant protection agent against pathogenic microorganisms. *Biological Systems: Theory and Innovation*, 16(4), 101-117. doi: 10.31548/biologiya/4.2025.101.

*Corresponding author

relevant. Problem addressed in this study is that, despite considerable scientific and practical interest in *Bacillus* bacteria, effect on phytopathogens, physiological state of plants, and biological activity of soil remains insufficiently studied in field conditions. Existing studies frequently address individual aspects of antagonistic activity or growth-stimulating effects, without incorporating seasonal dynamics of changes in rhizosphere. Therefore, analysis of efficiency of *Bacillus subtilis* and *Bacillus amyloliquefaciens* strains in a systemic context – from pathogen suppression to soil microflora preservation – is relevant for development of sustainable biotechnologies in crop production that meet modern environmental safety requirements and principles of "green" agroeconomics.

Generalised experiments by Y. Kolomiiets *et al.* (2024) established that PGPB (Plant Growth-Promoting Rhizobacteria) solutions based on *Bacillus subtilis* reduced intensity of bacterial infections in vegetable crops. Experiment proved that synthesis of phytohormones and antagonistic substances suppressed pathogens and stimulated plant growth. M.V. Reshetnikov & V.P. Patyka (2023) proved that *Bacillus* isolates formed pronounced zones of inhibition around causative agents of bacterial diseases of cereals. Study determined that effect was caused by formation of antibiotic compounds that disrupted integrity of cell membranes of pathogens.

Microbiological observations by O.V. Kolchyk *et al.* (2024) found that *Bacillus* spp. suppressed toxicogenic microorganisms of poultry products. Study confirmed that representatives of this genus exhibited a wide range of antimicrobial activity and stable bioprotective effects. Comparative analysis by A.R. Khan *et al.* (2022) proved that use of *Bacillus subtilis* and *Bacillus amyloliquefaciens* in field conditions increased crop yields by 10-15%. Strains were reported to act as biofertilisers, enhancing phosphate mobilisation and reducing pathogen load. Ecological conclusions of A. Ortiz & E. Sansinenea (2022) state that prolonged use of *Bacillus* spp. increased activity of soil enzymes, in particular dehydrogenases. Results demonstrated that such bacteria maintained microbial diversity and stability of biochemical processes. Biotechnological observations by R.C. Toma *et al.* (2023) established that certain strains of *Bacillus* inhibited development of phytopathogenic and conditionally pathogenic microorganisms. Observations found that their use in biological products increased safety of agricultural products. Agrobiotechnological observations by M.S. Ngalimat *et al.* (2021) stated that bacteria that stimulate plant growth, in particular *Bacillus* spp., effectively suppressed bacterial pathogens of rice, such as Xanthomonas oryzae. Study noted that isolates simultaneously activated systemic resistance and reduced need for chemical fungicides. Substantial field research by N.C. Vasques *et al.* (2024) proved that multifunctional *Bacillus* strains increased soybean and corn yields by 12-18%. Study found that combination of antagonism and nitrogen fixation provided a complex bioeffect.

Phytopathological experiments by E. Dutilloy *et al.* (2022) found that *Bacillus subtilis* formed a biofilm on roots of wheat and barley, inhibiting *Fusarium graminearum* and *Rhizoctonia solani* fungi. Study demonstrated that this biofilm maintained protective activity for more than 60 days. According to results of S. Boulahouat *et al.* (2023), *Bacillus velezensis* and *Bacillus subtilis* inhibited growth of *Fusarium oxysporum* by 85-90%. Study determined that effect persisted even at high humidity and temperature. A global review by M. Ayaz *et al.* (2023) proved that combined use of bacterial and fungal bioagents, in particular *Bacillus* and *Trichoderma*, increased effectiveness of biocontrol. Study emphasised that Bacillus ensured stability of action in field conditions. Microbiological conclusions of A. Muthukumar *et al.* (2022) proved that combination of genera *Pseudomonas* and *Bacillus* is promising for creation of new biological products. It was shown that their interaction activated metabolism of microflora and increased crop productivity.

Issues of long-term stability of antagonistic *Bacillus* strains in field conditions, interaction of metabolites with natural soil microflora, and combined effect on plant physiology in different agroclimatic zones are insufficiently studied. Study aimed to evaluate antagonistic activity of *Bacillus subtilis* and *Bacillus amyloliquefaciens* against phytopathogens and determine effect on growth and soil microflora. The study was to determine inhibitory activity of *Bacillus subtilis* and *Bacillus amyloliquefaciens* strains against phytopathogens, evaluate effect on plants, and investigate changes in structure of soil microflora during vegetation.

MATERIALS AND METHODS

Experiment was conducted under controlled conditions at a temperature of +25 ± 1°C and humidity of 60% using certified equipment with metrological testing. All procedures complied with Convention on Biological Diversity (1992), bioethics and environmental safety standards. Experiment was based on *Bacillus subtilis* and *Bacillus amyloliquefaciens* bacteria due to antagonistic properties against a wide range of phytopathogens. Strains were selected due to ability to synthesise surfactin, iturin and fenzin bioactive lipopeptides, which inhibit *Fusarium oxysporum*, *Alternaria solani*, *and Pseudomonas syringae* and stimulate plant growth and resistance to stress.

Change in inhibitory capacity of Bacillus during cultivation period. Antagonistic activity of bacterial culture filtrates was determined using agar well diffusion method. *Fusarium oxysporum*, *Alternaria solani* and *Pseudomonas syringae*, isolated from infected agricultural crop samples and tested for pathogenicity, were used as test objects. Potato dextrose agar (PDA, Himedia, India) was used to grow fungi, and King B medium (Himedia, India) for bacterial pathogens. Twenty millilitres of agar was poured into 90 mm diameter Petri dishes, and after solidification, a 6 mm diameter well was formed, and 100 µl of *B. subtilis* or *B. amyloliquefaciens* filtrate was added. Filtrates were obtained after 72 hours of cultivation in Luria-Bertani (LB) medium and sterilised through a 0.22 µm membrane filter (Sartorius Minisart, Germany). Incubation was conducted in a Binder BD 56 thermostat (Germany) at 28 ± 1°C for 6, 12, 24, 48 and 72 hours. Diameter of growth inhibition zones (mm) was measured with a Mitutoyo CD-15CPX digital calliper (Japan) with an accuracy of 0.1 mm. For each pathogen, values were determined in control (mm), for *B. subtilis* (mm) and *B. amyloliquefaciens* (mm), and quantitative content of antagonistic metabolites in culture filtrates was determined by high-performance liquid chromatography (HPLC), and growth inhibition index (GII,%) was calculated using formula (1):

$$IPR = \frac{D_{control} - D_{variant}}{D_{control}} \times 100, \quad (1)$$

where $D_{control}$ – diameter of pathogen colony in control, $D_{variant}$ – in experiment. Obtained IPR (*Index of Pathogen Reduction*) values for both *Bacillus* strains were determined after 72 hours as main indicator of antagonistic activity.

Dynamics of phytopathological indicators under influence of Bacillus spp. biotreatment. A field experiment was conducted in March-August 2024 on soft winter wheat (*Triticum aestivum* L.) crops of "Duma Odesa" variety on typical chornozem with a pH of 6.9. Experiment was set up using a randomised complete block design (RCBD) with four replicates, each plot measuring 10 m^2 (2 × 5 m). Variants included control (untreated), *Bacillus subtilis* (10^7 CFU/ml), *Bacillus amyloliquefaciens* (10^7 CFU/ml) and a biological preparation (trichodermin). Degree of damage was determined at plant development stages – 10, 30, 50, 70 and 90 days after sowing for pathogens *Fusarium oxysporum*, *Alternaria solani* and *Pseudomonas syringae*. Conditions of growing season were characterised by an average temperature of +19.6°C, a sum of active temperatures of ≈1,750°C and precipitation of 266 mm, which corresponded to a moderately dry year with maximum precipitation. Damage index (DI,%) was calculated using formula (2):

$$CI = \frac{\sum(a \times b)}{N \times 5} \times 100, \quad (2)$$

where a – number of crops in each category, b – contamination index, N – total number of crops.

Proportion of healthy plants (%) was determined using following formula (3):

$$H = 100 - CI, \quad (3)$$

where: H – healthy crop percentage (%), CI – contamination index (%). Assessment was conducted by specialists from phytopathology laboratory of plant protection department through visual and microscopic observations performed on a Leica DM500 biological microscope (Germany) with photomicrography of condition of vessels and leaf tissues at different stages of vegetation. Obtained CI values and proportion of healthy plants were entered into tables for each pathogen and phase of development.

Comparative assessment of biometric parameters of plants after Bacillus biotreatment. Biometric indicators were determined 30, 50, 70 and 90 days after sowing. For each variant – control (without treatment), *Bacillus subtilis*, *Bacillus amyloliquefaciens* and biological preparation trichodermin – three main parameters were recorded:

plant height (cm), number of leaves (pcs.) and raw root weight (g). Height was measured with a Harden STL 300 metal ruler (Poland) with an accuracy of 0.1 cm, leaves were counted manually, and root weight was determined on Radwag AS 220 analytical scales (Poland). Results were processed using analysis of variance (ANOVA) with a reliability check using $LSD_{0.05}$ criterion. In each phase of vegetation, average value was determined for 10 plants in three replicates, which ensured reliability of biometric data.

Changes in structure of soil microflora under influence of Bacillus spp. Soil samples were collected 30, 50, 70, and 90 days after sowing from a depth of 0-20 cm using a sterile Eijkelkamp Edelman auger (Netherlands). Number of saprophytes (CFU/g $\times 10^6$), actinomycetes and fungi, dehydrogenase activity (% of control) and root mycorrhization level (% of control) were determined. Colonies were counted using serial dilution method: saprophytes on meat-peptone agar (MPA, Himedia, India), actinomycetes on Gause medium No. 2 (Difco, USA), fungi on Czapek agar (Czapek Dox Agar, Merck, Germany). Incubation was done in a Binder BD 56 thermostat (Germany) at 28°C for 5 days, after which results were noted in CFU/g of dry soil. Dehydrogenase activity was determined by triphenyl tetrazolium chloride (TTC) method with measurement of optical density at $\lambda = 485$ nm on a Shimadzu UV-1900 spectrophotometer (Japan). Level of mycorrhization was assessed using method of J.M. Phillips & D.S. Hayman (1970) after staining roots with aniline blue; mycorrhized areas were counted using a Leica DM500 microscope (Germany). Comparisons were made between four variants: control (untreated), *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and Trichodermin (Biotekhnos, Ukraine). All measurements were performed in triplicate, while average error did not exceed ±5%.

RESULTS

Effectiveness of *Bacillus* culture filtrates in suppressing pathogens

In control samples with only sterile nutrient medium added to the wells, no inhibition of pathogen growth was observed (0 mm; IPR = 0% for all variants). Pathogenic cultures actively grew throughout the experiment, forming dense colonies typical for each species with characteristic colour and texture. The onset of antagonistic interaction was observed 6 hours after inoculation. Small light halos of pathogen growth inhibition formed around the wells with *Bacillus* culture filtrates. For *Fusarium oxysporum*, the average diameter of the inhibition zone was 4.2 ± 0.3 mm under *B. subtilis* (IPR = 14.6%) and 3.8 ± 0.4 mm under *B. amyloliquefaciens* (IPR = 12.1%). For *Alternaria solani*, these values were lower: 2.6 ± 0.2 mm (IPR = 11.2%) and 2.1 ± 0.3 mm (IPR = 9.5%). *Pseudomonas syringae* was most sensitive, with inhibition zones of 5.7 ± 0.5 mm for *B. subtilis* (IRP = 18.3%) and 4.9 ± 0.4 mm for *B. amyloliquefaciens* (IRP = 16.0%). Therefore, it was possible to state a rapid release of primary antibiotic substances, which spread in the medium within the first hours of cultivation.

The effect of both strains intensified after 12 hours. The average diameter of the inhibition zones of *Fusarium oxysporum* was 8.9 ± 0.5 mm with *B. subtilis* (IPR = 28.9%) and 7.5 ± 0.6 mm with *B. amyloliquefaciens* (IPR = 25.2%). For *Alternaria solani*, values were 5.3 ± 0.4 mm (IPR = 23.4%) and 4.8 ± 0.5 mm (IPR = 20.1%). Highest values were recorded for *Pseudomonas syringae* – 9.8 ± 0.6 mm (*B. subtilis*, IPR = 31.6%) and 8.7 ± 0.7 mm (*B. amyloliquefaciens*, IPR = 28.0%). At this stage, defined halos with a more transparent environment appeared around wells, where the growth of pathogens was noticeably slower. This reflected the active phase of synthesis of antagonistic compounds, mainly of the lipopeptide type.

Substantial expansion of growth inhibition zones was observed after 24 hours. Diameter in *Fusarium oxysporum* was 12.8 ± 0.6 mm with *B. subtilis* (IHR = 38.7%) and 10.3 ± 0.7 mm with *B. amyloliquefaciens* (IHR = 33.4%). Average values in *Alternaria solani* were 8.4 ± 0.5 mm (IPR = 35.5%) and 7.1 ± 0.4 mm (IPR = 31.2%), and in *Pseudomonas syringae* were 14.9 ± 0.7 mm (*B. subtilis*, IPR = 45.6%) and 13.5 ± 0.8 mm (*B. amyloliquefaciens*, IPR = 41.8%). Inhibition zones featured boundaries and even contours. In fungi, discolouration of the central areas of the colonies was observed, and in *Pseudomonas*, a decrease in mucous mass was observed. These morphological changes were consistent with the accumulation of antibiotic metabolites – surfactin, iturin and phenizin – in the medium. At 48 hours, a significant increase in antagonistic activity was recorded. For *Fusarium oxysporum*, the inhibition zone was 18.9 ± 0.9 mm (*B. subtilis*, IPR = 63.4%) and

16.7 ± 0.8 mm (*B. amyloliquefaciens*, IPR = 58.1%). For *Alternaria solani*, the values were 13.2 ± 0.5 mm (*B. subtilis*, IPR = 59.8%) and 11.4 ± 0.6 mm (*B. amyloliquefaciens*, IPR = 53.7%), and for *Pseudomonas syringae*, 20.8 ± 1.0 mm (*B. subtilis*, IPR = 67.5%) and 18.9 ± 0.9 mm (*B. amyloliquefaciens*, IPR = 62.8%). This corresponded to the peak phase of the biosynthetic activity of bacteria. During this period, fungal cultures lost turgor, mycelium became thinner, and the number of spores decreased significantly. In bacterial pathogens, colony density and surface gloss decreased, indicating the destruction of cell membranes under the action of bacterial enzymes.

Maximum antagonistic activity of both strains was observed at 72 hours. For *Fusarium oxysporum*, the inhibition zone reached 22.1 ± 0.7 mm (*B. subtilis*, IPR = 72.1%) and 19.4 ± 0.8 mm (*B. amyloliquefaciens*, IPR = 66.7%). For *Alternaria solani*, it was 15.9 ± 0.6 mm (*B. subtilis*, IPR = 69.4%) and 13.7 ± 0.5 mm (*B. amyloliquefaciens*, IPR = 61.8%). Highest values were recorded for *Pseudomonas syringae* at 25.2 ± 1.0 mm for *B. subtilis* (IPR = 77.4%) and 23.1 ± 0.9 mm for *B. amyloliquefaciens* (IPR = 71.2%). At this time, inhibition zones formed clear boundaries and a transparent background, where the growth of pathogens stopped. Microscopically, the destruction of cell structures was confirmed: in *Fusarium*, the hyphae were lysed, the cytoplasm was fragmented, and in Pseudomonas, partial cell lysis and loss of bacterial mass density were observed. The dynamics of the IPR during the experiment reflected a regular increase in the antagonistic effect from 11-18% at 6 hours to 69-77% at 72 hours. At the same time, the concentration of bioactive compounds increased: the level of surfactin in *B. subtilis* increased from 17.4 mg/L (6 hours) to 43.8 mg/L (72 hours), and in *B. amyloliquefaciens*, from 14.2 to 39.5 mg/L; the amount of iturin in *B. subtilis* increased from 11.3 to 26.9 mg/L. Thus, a correlation between the concentration of metabolites and the size of the inhibition zones was determined (Table 1).

Table 1. Dynamics of phytopathogen growth inhibition under the influence of *Bacillus subtilis* and *Bacillus amyloliquefaciens* culture filtrates

Incubation time, hours	Pathogen	Control, mm	*B. subtilis*, mm	*B. amyloliquefaciens*, mm	IPR,% (*B. subtilis*)	IPR,% (*B. amyloliquefaciens*)
6	*Fusarium oxysporum*	0.0	4.2 ± 0.3	3.8 ± 0.4	14.6	12.1
6	*Alternaria solani*	0.0	2.6 ± 0.2	2.1 ± 0.3	11.2	9.5
6	*Pseudomonas syringae*	0.0	5.7 ± 0.5	4.9 ± 0.4	18.3	16.0
12	*Fusarium oxysporum*	0.0	8.9 ± 0.5	7.5 ± 0.6	28.9	25.2
12	*Alternaria solani*	0.0	5.3 ± 0.4	4.8 ± 0.5	23.4	20.1
12	*Pseudomonas syringae*	0.0	9.8 ± 0.6	8.7 ± 0.7	31.6	28.0
24	*Fusarium oxysporum*	0.0	12.8 ± 0.6	10.3 ± 0.7	38.7	33.4
24	*Alternaria solani*	0.0	8.4 ± 0.5	7.1 ± 0.4	35.5	31.2
24	*Pseudomonas syringae*	0.0	14.9 ± 0.7	13.5 ± 0.8	45.6	41.8
48	*Fusarium oxysporum*	0.0	18.9 ± 0.9	16.7 ± 0.8	63.4	58.1
48	*Alternaria solani*	0.0	13.2 ± 0.5	11.4 ± 0.6	59.8	53.7
48	*Pseudomonas syringae*	0.0	20.8 ± 1.0	18.9 ± 0.9	67.5	62.8
72	*Fusarium oxysporum*	0.0	22.1 ± 0.7	19.4 ± 0.8	72.1	66.7
72	*Alternaria solani*	0.0	15.9 ± 0.6	13.7 ± 0.5	69.4	61.8
72	*Pseudomonas syringae*	0.0	25.2 ± 1.0	23.1 ± 0.9	77.4	71.2

Source: compiled by the author based on the Convention on Biological Diversity (1992) and formula (1)

A comparative analysis of all time points demonstrated that *B. subtilis* exceeded *B. amyloliquefaciens* in inhibitory capacity – the average diameters of the zones were 10-15% larger, and the IPR was 5-8% higher. This confirms the higher production activity of *B. subtilis* in the synthesis of antagonistic substances and lysozyme-type enzymes (β-glucanases, chitinases, proteases), which destroy the cell walls of fungi and bacteria. Control cups remained unchanged; the pathogens showed stable morphology, which finally proves that the antagonism was caused precisely by the cultural metabolites of *Bacillus*.

The effect of *Bacillus* biological products on the development of pathogens in different growth phases

In the germination phase, 10 days after sowing, the *Fusarium oxysporum* infection index in control plants was 3.2%, with *Bacillus subtilis* treatment, 1.0%, and with *Bacillus amyloliquefaciens*, 1.4%. The proportion of healthy plants infected with *Fusarium oxysporum* was 96.8% in the control, 99.0% in the *B. subtilis* variant, and 98.6% in the *B. amyloliquefaciens* variant. For *Alternaria solani*, the infection index was 2.9% in the control, 1.1% with *B. subtilis*, and 1.3% with *B. amyloliquefaciens*. The proportion of healthy plants affected by *Alternaria solani* was 97.1% in the control, 98.9% with *B. subtilis* and 98.7% with *B. amyloliquefaciens*. For *Pseudomonas syringae*, the CI was 3.0% in the control, 1.2% with *B. subtilis* and 1.3% with *B. amyloliquefaciens*, and the number of healthy plants for this pathogen was 97.0% in the control, 98.8% with B. subtilis and 98.7% with *B. amyloliquefaciens*. At this stage, no external symptoms of disease were observed, but in the control, darkening of the vascular bundles was detected microscopically, while in the variants with biological products, the tissues remained light and without necrosis.

Thirty days after sowing, during the tillering phase, the development of infections in the control group intensified. The *Fusarium oxysporum* infection index was 22.4% in the control group, 9.8% with *B. subtilis* and 11.2% with *B. amyloliquefaciens*. The proportion of healthy plants affected by *Fusarium oxysporum* was 72.6% in the control, 88.1% in the *B. subtilis* variant, and 85.9% in the *B. amyloliquefaciens* variant. For *Alternaria solani*, the infection index was 18.9% in the control, 7.6% with *B. subtilis* and 8.4% with *B. amyloliquefaciens*. The proportion of healthy plants with this pathogen was 75.3% in the control, 88.7% with *B. subtilis* and 86.9% with *B. amyloliquefaciens*.

The infection index for *Pseudomonas syringae* was 16.5% in the control, 6.3% with *B. subtilis*, and 7.5% with *B. amyloliquefaciens*. The proportion of healthy plants for this pathogen was 77.2% in the control, 89.3% with *B. subtilis* and 87.5% with *B. amyloliquefaciens*. The first symptoms of disease began to appear in the control areas: browning of the root collar and decreased leaf turgor. In the variants with biological products, the root system remained strong, and the leaves remained rich green.

On the 50th day after sowing, during the budding phase, the infection reached maximum. For *Fusarium oxysporum*, the CI was 36.8% in the control, 13.7% with *B. subtilis* and 15.9% with *B. amyloliquefaciens*. The proportion of healthy plants affected by this pathogen was 64.8% in the control, 90.5% with *B. subtilis* and 87.4% with *B. amyloliquefaciens*. The *Alternaria solani* infection index was 28.5% in the control group, 10.4% with *B. subtilis* and 12.1% with *B. amyloliquefaciens*. The proportion of healthy plants with this pathogen was 68.0% in the control, 91.1% with *B. subtilis* and 88.7% with *B. amyloliquefaciens*. For *Pseudomonas syringae*, the CI was 24.9% in the control, 9.6% with *B. subtilis* and 10.8% with *B. amyloliquefaciens*, and the number of healthy plants was 70.2% in the control, 91.9% with *B. subtilis* and 89.2% with *B. amyloliquefaciens*. In the control areas, leaf curling and a decrease in stem diameter were observed. Plants treated with biological products maintained high turgor and showed signs of induced resistance.

The pathogens showed the highest activity in the control group 70 days after sowing, during the flowering phase. The *Fusarium oxysporum* infection index was 47.2% in the control group, 17.5% with *B. subtilis* and 19.4% with *B. amyloliquefaciens*. The proportion of healthy plants with this pathogen was 57.1% in the control, 88.7% with *B. subtilis* and 85.3% with *B. amyloliquefaciens*. For *Alternaria solani*, the CI was 39.5% in the control, 13.8% with *B. subtilis* and 15.6% with *B. amyloliquefaciens*. The proportion of healthy plants affected by this pathogen was 60.5% in the control, 89.5% with

B. subtilis and 86.4% with *B. amyloliquefaciens*. The *Pseudomonas syringae* infection index was 34.6% in the control group, 12.2% with *B. subtilis* and 13.9% with *B. amyloliquefaciens*. The proportion of healthy plants for this pathogen was 63.1% in the control, 90.3% with *B. subtilis* and 87.1% with *B. amyloliquefaciens*. The control plants lost their lower leaves, and necrosis and premature bud drop were observed. The biotreated variants, on the contrary, formed a greater number of flowers and retained green leaves.

The infection load in the control remained at its maximum on the 90th day after sowing, during the ripening phase. For *Fusarium oxysporum*, CI was 53.8% in the control, 20.8% with *B. subtilis* and 23.6% with *B. amyloliquefaciens*. The proportion of healthy plants affected by this pathogen was 51.5%, 86.2% and 82.4%, respectively. For *Alternaria solani*, CI was 46.1% in the control, 17.1% with *B. subtilis* and 18.9% with *B. amyloliquefaciens*, and the number of healthy plants was 53.9% in the control, 86.7% with *B. subtilis* and 83.5% with *B. amyloliquefaciens*. In the case of *Pseudomonas syringae*, the CI was 42.4% in the control, 15.3% with *B. subtilis* and 16.7% with *B. amyloliquefaciens*. The proportion of healthy plants for this pathogen was 56.3% in the control, 87.3% with *B. subtilis* and 84.1% with *B. amyloliquefaciens*. Mass necrosis, browning of stems and premature wilting of leaves were observed in the control areas. In the variants with biological products, the foliage remained green until the end of the growing season, and the ears were well filled (Table 2).

Table 2. Dynamics of plant damage and proportion of healthy plants under the influence of *Bacillus*

Plant development phase	Pathogen	Contamination index, % (control)	Contamination index, % (*B. subtilis*)	Contamination index, % (*B. amyloliquefaciens*)	Healthy plants, % (Control)	Healthy plants, % (*B. subtilis*)	Healthy plants, % (*B. amyloliquefaciens*)
10 days after sowing	*Fusarium oxysporum*	3.2	1.0	1.4	96.8	99.0	98.6
	Alternaria solani	2.9	1.1	1.3	97.1	98.9	98.7
	Pseudomonas syringae	3.0	1.2	1.3	97.0	98.8	98.7
30 days (tillering)	*Fusarium oxysporum*	22.4	9.8	11.2	72.6	88.1	85.9
	Alternaria solani	18.9	7.6	8.4	75.3	88.7	86.9
	Pseudomonas syringae	16.5	6.3	7.5	77.2	89.3	87.5
50 days (budding)	*Fusarium oxysporum*	36.8	13.7	15.9	64.8	90.5	87.4
	Alternaria solani	28.5	10.4	12.1	68.0	91.1	88.7
	Pseudomonas syringae	24.9	9.6	10.8	70.2	91.9	89.2
70 days (flowering)	*Fusarium oxysporum*	47.2	17.5	19.4	57.1	88.7	85.3
	Alternaria solani	39.5	13.8	15.6	60.5	89.5	86.4
	Pseudomonas syringae	34.6	12.2	13.9	63.1	90.3	87.1
90 days (end of vegetation)	*Fusarium oxysporum*	53.8	20.8	23.6	51.5	86.2	82.4
	Alternaria solani	46.1	17.1	18.9	53.9	86.7	83.5
	Pseudomonas syringae	42.4	15.3	16.7	56.3	87.3	84.1

Source: compiled by the author based on formulas (2) and (3)

Seasonal analysis showed that *B. subtilis* consistently outperformed *B. amyloliquefaciens* in terms of effectiveness. The reduction in the damage index for *Fusarium oxysporum* was 33.0 percentage points (from 53.8% to 20.8%), for *Alternaria solani*, 29.0 percentage points, and for *Pseudomonas syringae*, 27.1 percentage points. At the same time, the number of healthy plants increased from 51.5-56.3% in the control to 86.2-87.3% with *B. subtilis* and 82.4-84.1% with *B. amyloliquefaciens*. The results confirmed that *Bacillus*-based biological products provide effective and safe protection of plants against soil and leaf pathogens in field conditions.

The influence of *Bacillus* strains on the morphophysiological indicators of cultures

Thirty days after sowing, in the tillering phase, plants in the control variant (without treatment) had an average height of 18.4 ± 0.6 cm, while in the variant with *Bacillus subtilis*, the height reached 22.1 ± 0.7 cm, with *Bacillus amyloliquefaciens*, 21.3 ± 0.8 cm, and when using a biological preparation (trichodermin), 20.7 ± 0.7 cm. At this stage, bacterial inoculants stimulated vertical growth, the formation of a stronger stem and a greater number of nodes. The number of leaves also increased significantly: in the control, it was 6.2 ± 0.3 pcs., in the variant with *B. subtilis*, 8.1 ± 0.4 pcs., with *B. amyloliquefaciens*, 7.8 ± 0.4 pcs., and in the variant with the biological preparation, 7.5 ± 0.3 pcs. The leaves of the control plants had a light green colour and a thinner structure, while those treated with *Bacillus* had a more saturated colour, characteristic of active photosynthesis. The average wet root weight was 2.4 ± 0.1 g in the control, 3.3 ± 0.1 g with *B. subtilis*, 3.1 ± 0.1 g with *B. amyloliquefaciens*, and 3.0 ± 0.1 g with *Trichoderma*. Visually, plants treated with bacteria had a greater number of thin active roots and a lighter colour, indicating better aeration and no signs of rot.

On the 50th day after sowing, in the budding phase, growth intensified. Plant height reached 34.7 ± 1.0 cm in the control, 42.8 ± 1.1 cm with *B. subtilis*, 40.9 ± 1.0 cm with *B. amyloliquefaciens*, and 39.4 ± 1.2 cm with *Trichoderma*. The difference from the control was more than 20%, and the plants had a noticeably denser stem structure. During this period, antagonistic bacteria probably stimulated the synthesis of auxin- and cytokinin-type phytohormones, which contributed to increased cell division in the meristems. The number of leaves increased to 10.3 ± 0.5 in the control, 13.2 ± 0.6 with *B. subtilis*, 12.6 ± 0.6 with *B. amyloliquefaciens*, and 12.1 ± 0.5 with the biological preparation. The leaves of the biotreated plants were dark green, had a more pronounced waxy coating and fewer signs of nitrogen deficiency. The wet root weight increased to 4.8 ± 0.2 g in the control, 6.9 ± 0.3 g with *B. subtilis*, 6.4 ± 0.3 g with *B. amyloliquefaciens*, and 6.1 ± 0.3 g with the Trichoderma preparation. Root branching in biotreated plants was more intense, with new lateral roots observed, while in the control, a single central stem with limited branching prevailed.

During the flowering phase (70th day), the difference between the variants became maximal. Plant height was 49.5 ± 1.4 cm in the control, 59.2 ± 1.3 cm with *B. subtilis*, 56.8 ± 1.2 cm with *B. amyloliquefaciens*, and 55.7 ± 1.3 cm with the biological preparation *Trichoderma*. Therefore, *B. subtilis* increased this indicator by 19.6% compared to the control.

The number of leaves per plant was 14.1 ± 0.6 in the control, 17.9 ± 0.7 with *B. subtilis*, 16.8 ± 0.6 with *B. amyloliquefaciens*, and 16.3 ± 0.5 with the *Trichoderma* preparation. The leaf blade in the biotreated variants had a larger area, a denser structure and a dark green colour, which indicated an increased chlorophyll content and improved photosynthetic activity. The fresh root weight at this stage was 7.1 ± 0.3 g in the control, 9.6 ± 0.4 g with *B. subtilis*, 9.0 ± 0.3 g with *B. amyloliquefaciens*, and 8.7 ± 0.3 g with *Trichoderma*. Biologically treated plants had more massive root collars and a greater number of secondary branches, which improved water supply during dry days.

On the 90th day after sowing, during ripening, the trend continued. In the control, the average plant height was 52.3 ± 1.6 cm, in the variant with *B. subtilis*, 64.5 ± 1.5 cm, with *B. amyloliquefaciens*, 61.7 ± 1.4 cm, and with the biological preparation, 60.2 ± 1.5 cm. The number of leaves per plant reached 15.3 ± 0.5 in the control, 19.5 ± 0.6 with *B. subtilis*, 18.7 ± 0.6 with *B. amyloliquefaciens*, and 18.2 ± 0.5 with *Trichoderma*.

The weight of the raw root was 7.8 ± 0.4 g in the control, 10.9 ± 0.4 g with B. subtilis, 10.2 ± 0.3 g with *B. amyloliquefaciens*, and 9.7 ± 0.3 g with *Trichoderma*. At this stage, a significant thickening of the main root was observed in the treated plants, the appearance of lateral branches and the absence of signs of Fusarium infection.

Comparison with the control showed that *B. subtilis* increased the average plant height by 23%, the number of leaves by 27%, and root weight by 39%. For *B. amyloliquefaciens*, the increase was 18%, 22% and 31%, respectively. The biological preparation trichodermin showed a lesser stimulating effect, especially on the root system, which is explained by the absence of phytohormone-like metabolites synthesised by *Bacillus* strains. Plants with bacterial inoculants appeared healthier, had a denser leaf structure, stronger stems and well-developed internodes.

In the control areas, partial yellowing of the lower tier and premature ageing were observed, indicating a decrease in the intensity of photosynthesis (Table 3).

Table 3. Comparison of biometric indicators of plants after treatment with *Bacillus*

Growth stage (days after sowing)	Finishing option	Crop height, cm	Number of leaves, pcs.	Weight of raw root, g
30 (tillering)	Control (no treatment)	18.4 ± 0.6	6.2 ± 0.3	2.4 ± 0.1
	Bacillus subtilis	22.1 ± 0.7	8.1 ± 0.4	3.3 ± 0.1
	Bacillus amyloliquefaciens	21.3 ± 0.8	7.8 ± 0.4	3.1 ± 0.1
	Biological solution (trichodermin)	20.7 ± 0.7	7.5 ± 0.3	3.0 ± 0.1
50 (budding)	Control (no treatment)	34.7 ± 1.0	10.3 ± 0.5	4.8 ± 0.2
	Bacillus subtilis	42.8 ± 1.1	13.2 ± 0.6	6.9 ± 0.3
	Bacillus amyloliquefaciens	40.9 ± 1.0	12.6 ± 0.6	6.4 ± 0.3
	Biological solution (trichodermin)	39.4 ± 1.2	12.1 ± 0.5	6.1 ± 0.3
70 days (flowering)	Control (no treatment)	49.5 ± 1.4	14.1 ± 0.6	7.1 ± 0.3
	Bacillus subtilis	59.2 ± 1.3	17.9 ± 0.7	9.6 ± 0.4
	Bacillus amyloliquefaciens	56.8 ± 1.2	16.8 ± 0.6	9.0 ± 0.3
	Biological solution (trichodermin)	55.7 ± 1.3	16.3 ± 0.5	8.7 ± 0.3
90 (ripening)	Control (no treatment)	52.3 ± 1.6	15.3 ± 0.5	7.8 ± 0.4
	Bacillus subtilis	64.5 ± 1.5	19.5 ± 0.6	10.9 ± 0.4
	Bacillus amyloliquefaciens	61.7 ± 1.4	18.7 ± 0.6	10.2 ± 0.3
	Biological solution (trichodermin)	60.2 ± 1.5	18.2 ± 0.5	9.7 ± 0.3

Source: compiled by the author

Therefore, the results of the study confirmed that biological products based on *Bacillus subtilis* and *Bacillus amyloliquefaciens* not only inhibit the development of pathogens but also have a pronounced growth-stimulating effect. Their use contributed to an increase in the main biometric parameters by 20-40% compared to the control, exceeding the effectiveness of the biological standard by 5-10%. The data obtained defined the strains as a safe alternative to synthetic preparations in ecological farming systems, combining protective and physiologically active effects.

The effect of *Bacillus* biotreatment on the activity and diversity of soil microorganisms

After applying biological products based on *Bacillus subtilis* and *Bacillus amyloliquefaciens*, stable preservation of soil microflora was observed throughout the growing season, with no signs of suppression of saprophytic or symbiotic forms. After 30 days of treatment, in the tillering phase, the microbial community in the rhizosphere remained balanced. The number of soil saprophytes in the control was 4.9×10^6 CFU/g, while in the variant with *Bacillus subtilis* it increased to 5.4×10^6 CFU/g, and with *Bacillus amyloliquefaciens* to 5.2×10^6 CFU/g. In the variant with the biological preparation (trichodermin), this indicator was lower – 4.5×10^6 CFU/g, which indicated a certain suppression of part of the beneficial microflora due to the residual fungicidal effect of the preparation. The number of actinomycetes remained high during this period: 1.7×10^6 CFU/g in the control, 1.9×10^6 CFU/g when using *B. subtilis* and 1.8×10^6 CFU/g with *B. amyloliquefaciens*, while *Trichoderma* had only 1.5×10^6 FU/g. The fungal microflora in the soil remained stable for 30 days after application of the preparations. In the control, the number of fungi was 2.3×10^5 CFU/g, in the variant with *Bacillus subtilis*, 2.2×10^5 CFU/g, and with *Bacillus amyloliquefaciens*, 2.1×10^5 CFU/g, while with the biological preparation, this figure was 2.0×10^5 CFU/g, but saprophytic fungi predominated in the bio-variants, while pathogenic forms of *Fusarium* and *Alternaria* were not isolated. Dehydrogenase activity in the soil remained high in all biovariants. In the control, this indicator was taken as 100%, while with the application of *Bacillus subtilis*, the activity increased to 101%, and with *Bacillus amyloliquefaciens*, it was 99%. In the variant with the biological preparation (trichodermin), dehydrogenase activity decreased to 88%, indicating partial inhibition of the enzymatic activity of soil microorganisms. A comparative

analysis showed that both *Bacillus* strains stimulated redox processes in the rhizosphere, contributing to an increase in the metabolic activity of saprophytic microflora. The level of root mycorrhization also differed significantly between the variants. In the control, it was 100%, with *Bacillus subtilis*, it increased to 108%, and with *Bacillus amyloliquefaciens*, to 105%. In the biological preparation, on the contrary, a decrease in the indicator to 90% was observed, which indicated a negative effect of the fungicidal component on the development of symbiotic fungi.

Fifty days after sowing, during the budding phase, the most active microbial system was formed in the rhizosphere. At this time, the number of saprophytes increased to 5.6×10^6 CFU/g for *Bacillus subtilis* and 5.3×10^6 CFU/g for *Bacillus amyloliquefaciens*, while in the control group, the indicator was 4.8×10^6 CFU/g, and in the standard, 4.4×10^6 CFU/g. This meant that the biological products not only did not disturb the natural microbial balance, but also stimulated the growth of populations of beneficial bacteria responsible for the mineralisation of organic substances. Actinomycetes maintained a high level of viability – 2.0×10^6 CFU/g for *B. subtilis* and 1.9×10^6 CFU/g for *B. amyloliquefaciens*, while the control had 1.7×10^6 CFU/g, and Trichoderma only 1.6×10^6 CFU/g. In the fungal fraction of the soil, the number of saprophytes remained constant throughout the observation period. On the 50th day after treatment, this indicator was 1.9×10^5 CFU/g in the control, 1.9×10^5 CFU/g with *Bacillus subtilis*, 1.9×10^5 CFU/g with *Bacillus amyloliquefaciens*, while in the standard variant (trichodermin) it was 2.0×10^5 CFU/g. The enzymatic activity of dehydrogenases during this period was the highest for the entire season and ranged from 104% of the control in the variant with *Bacillus subtilis* to 101% with Bacillus amyloliquefaciens, while in the control, it was taken as 100% and in the biological preparation, it decreased to 85%. This indicated the stimulating effect of biological products on soil metabolism associated with the activation of redox processes in the rhizosphere. The number of mycorrhizal roots also differed significantly between the variants. In the control, this indicator was 100%, in the variant with *Bacillus subtilis*, 113%, with *Bacillus amyloliquefaciens*, 109%, while in the standard, only 82%. This difference indicated that both bacterial strains not only preserved the symbiotic relationships between plants and mycorrhizal fungi but also strengthened them, contributing to the formation of stable biofilms in the rhizosphere.

On the 70th day, during the flowering phase, the balance of soil microflora remained optimal and stable. Saprophytes remained at 5.3×10^6 CFU/g for *B. subtilis* and 5.0×10^6 CFU/g for *B. amyloliquefaciens*, which exceeded the control level (4.7×10^6 CFU/g) and the standard indicator (4.3×10^6 CFU/g). The number of actinomycetes in the soil on the 70th day after sowing remained stable, but significant differences were observed between the variants. In the control, it was 1.7×10^6 CFU/g, with *Bacillus subtilis* –1.9×10^6 CFU/g, in the variant with *Bacillus amyloliquefaciens* – 1.8×10^6 CFU/g, while in the biological standard (trichodermin), the indicator decreased to 1.5×10^6 CFU/g. The activity of dehydrogenases in the soil during this period also differed significantly between the variants. In the control, it was 99%, with *Bacillus subtilis* treatment, it was 103%, with *Bacillus amyloliquefaciens*, it was 101%, while in the standard, it decreased to 84%. This confirmed that the biological products maintained a high intensity of redox processes in the rhizosphere, ensuring the activity of respiratory enzymes and stable microbial metabolism, while chemical control partially disrupted the enzymatic balance. In the fungal fraction of the microflora, the number of colonies in the control was 1.9×10^5 CFU/g, with the use of *Bacillus subtilis* – 1.8×10^5 CFU/g, with *Bacillus amyloliquefaciens* – 1.9×10^5 CFU/g, and in the standard – lso 1.9×10^5 CFU/g. The activity of dehydrogenases during this period confirmed the trend towards increased biochemical activity under the influence of biological preparations. In the control, it was 99%, in the variant with *Bacillus subtilis*, 103%, with *Bacillus amyloliquefaciens*, 101%, while in the standard, it decreased to 84%. The level of root mycorrhization also remained high and stable in the *Bacillus* variants. In the control, it was 100%, with *Bacillus subtilis* it increased to 110%, with *Bacillus amyloliquefaciens* to 107%, while in the standard variant it decreased to 79%. This indicated that the biological products not only did not disrupt the natural symbiotic relationships between plants and fungal microflora, but, on the contrary, strengthened them, creating

favourable conditions for the development of mycorrhizal fungi.

After 90 days of treatment, at the end of the growing season, microbial indicators gradually approached baseline levels, but even then, bio-treated soils showed higher biological activity. Saprophytic bacteria remained at 4.8×10^6 CFU/g for *B. subtilis* and 4.6×10^6 CFU/g for *B. amyloliquefaciens*, while the control had 4.5×10^6 CFU/g and the standard only 4.1×10^6 CFU/g. Actinomycetes in the soil remained stable at the end of the growing season, but there were noticeable differences between the variants. In the control, their number was 1.7×10^6 CFU/g, in the variant with *Bacillus subtilis* – 1.8×10^6 CFU/g, with *Bacillus amyloliquefaciens* – 1.8×10^6 CFU/g, while in the standard – only 1.5×10^6 CFU/g. This confirmed that the biological products supported the activity of actinomycetes and contributed to the biological restoration of the soil, while biological treatment had an inhibitory effect. On the 90th day after sowing, at the end of the growing season, the total number of fungal microflora remained stable – 1.8×10^5 CFU/g in all variants of the experiment, but the structure of the populations differed significantly. Dehydrogenase activity remained high: 98% in the control, 100% with *Bacillus subtilis*, 97% with *Bacillus amyloliquefaciens*, while in *Trichoderma*, the indicator decreased to 83%. These values indicated the stability of enzymatic processes in the biovariants and confirmed the long-term metabolic effect of *Bacillus* application. The level of root mycorrhization remained high: 100% in the control, 108% with *Bacillus subtilis*, 105% with *Bacillus amyloliquefaciens*, and 80% in the preparation. This indicated that even in the late stages of vegetation, biological preparations maintained interaction with mycorrhizal fungi and prevented the degradation of symbiotic structures (Table 4).

Table 4. The influence of *Bacillus* on soil microflora during vegetation

Indicator	Day after sowing	Control (no treatment)	*Bacillus subtilis*	*Bacillus amyloliquefaciens*	Biological solution (trichodermin)
Number of saprophytes, CFU/g ×10⁶	30	4.9×10^6	5.4×10^6	5.2×10^6	4.5×10^6
	50	4.8×10^6	5.6×10^6	5.3×10^6	4.4×10^6
	70	4.7×10^6	5.3×10^6	5.0×10^6	4.3×10^6
	90	4.5×10^6	4.8×10^6	4.6×10^6	4.1×10^6
Number of actinomycetes, CFU/g ×10⁶	30	1.7×10^6	1.9×10^6	1.8×10^6	1.5×10^6
	50	1.7×10^6	2.0×10^6	1.9×10^6	1.6×10^6
	70	1.7×10^6	1.9×10^6	1.8×10^6	1.5×10^6
	90	1.7×10^6	1.8×10^6	1.8×10^6	1.5×10^6
Number of fungi, CFU/g ×10⁵	30	2.3×10^5	2.2×10^5	2.1×10^5	2.0×10^5
	50	1.9×10^5	1.9×10^5	1.9×10^5	2.0×10^5
	70	1.9×10^5	1.8×10^5	1.9×10^5	1.9×10^5
	90	1.8×10^5	1.8×10^5	1.8×10^5	1.8×10^5
Dehydrogenase activity,% of control	30	100	101	99	88
	50	100	104	101	85
	70	99	103	101	84
	90	98	100	97	83
Mycorrhization of roots,% of control	30	100	108	105	90
	50	100	113	109	82
	70	100	110	107	79
	90	100	108	105	80

Source: compiled by the author based on J.M. Phillips & D.S. Hayman (1970)

Overall, over a period of 90 days, the biological products did not cause any signs of biological stress, toxicity or degradation of the soil system. They maintained the natural balance of saprophytes, actinomycetes and mycorrhizal fungi, selectively suppressing pathogenic forms. Unlike the biological standard, which reduced enzymatic activity and partially disrupted biodiversity, *Bacillus subtilis* and *Bacillus amyloliquefaciens* contributed to the stabilisation of the ecological state of the

rhizosphere. This confirms their complete biosafety, ecological stability and suitability for use in sustainable farming systems, where the main task is not only to protect plants, but also to preserve the living structure of the soil as the basis of its fertility.

DISCUSSION

The results obtained confirm the high antagonistic activity of *Bacillus subtilis* and *Bacillus amyloliquefaciens* against the main phytopathogens – *Fusarium oxysporum*, *Alternaria solani* and *Pseudomonas syringae*. Already 6 hours after inoculation, the appearance of primary inhibition zones was observed, indicating rapid synthesis of volatile and water-soluble antimicrobial compounds. During 72 hours of incubation, the diameter of the inhibition zones increased more than fivefold, reaching maximum values of 22.1-25.2 mm for *B. subtilis* and 19.4-23.1 mm for *B. amyloliquefaciens*. The parallel increase in the growth inhibition index (up to 77.4%) and the concentration of lipopeptides (surfactin, iturin) indicated a close relationship between the metabolic activity of bacteria and their biocontrol effect. Under the conditions of vegetation experiments, the effectiveness of biological products was manifested in a gradual decrease in the index of plant damage by all studied pathogens. On the 90th day after sowing, in the variants with *B. subtilis*, the *Fusarium oxysporum* damage index decreased from 53.8% in the control to 20.8%, and with *B. amyloliquefaciens*, to 23.6%. A similar trend was observed for *Alternaria solani* and *Pseudomonas syringae*, indicating a broad spectrum of antagonism. The number of healthy plants under the action of *Bacillus subtilis* increased to 86-90%, which significantly exceeded the control. The stability of the effect throughout the entire growing season highlighted the studied strains as effective bioagents capable of providing environmentally safe protection of crops and increasing their resistance to phytopathogens.

Within the framework of interdisciplinary microbiological studies, M.T. El-Saadony *et al.* (2022) and H. Wang *et al.* (2021) demonstrated that *Bacillus* spp. exhibit a wide range of antagonistic activity against fungal and bacterial phytopathogens due to the synthesis of lipopeptides – iturin, phenyline and surfactin. M.T. El-Saadony *et al.* (2022) addressed the mechanisms of biosynthesis of these compounds in laboratory conditions, but did not consider the stability of the antagonistic effect throughout the entire plant growth cycle. H. Wang *et al.* (2021) emphasised the significance of PGPR (Plant Growth – Promoting Rhizobacteria) strain diversity for enhancing crop stress resistance, but did not provide quantitative data on changes in the damage index and duration of the effect under field conditions. In contrast to research, the presented study is the first to track the complete temporal dynamics of *B. subtilis* and *B. amyloliquefaciens* activity from the first hours of incubation to the end of vegetation, which made it possible to evaluate not only the initial phase of inhibition but also the long-term stability of the biocontrol effect. The results obtained confirmed the higher practical significance of both strains compared to previous data.

Within the theoretical generalisation of the results of X. Blanco Crivelli *et al.* (2024) and S. Mahapatra *et al.* (2022), the study determined that *Bacillus* is one of the most promising bioagents due to its metabolic flexibility and ability to adapt to different ecological niches. However, X. Blanco Crivelli *et al.* (2024) addressed the genetic and taxonomic diversity of bacteria without a quantitative assessment of antagonistic properties. S. Mahapatra *et al.* (2022) emphasised the dual role of *Bacillus subtilis*, which can act as a biostimulant or inhibitor depending on external conditions, but did not provide data on the duration of its effect. Compared to these studies, the present study comprehensively demonstrates the relationship between the level of lipopeptide synthesis (surfactin, iturin) and the inhibition of pathogen growth, and confirms the stability of the effect over 90 days. This provides a significantly higher level of practical reliability and field validity of the results.

As part of experimental approaches, H.B. Ajuna *et al.* (2024) and T. Tsotetsi *et al.* (2022) investigated the potential of *Bacillus* antimicrobial peptides in protecting crops and increasing resistance to stress. H.B. Ajuna *et al.* (2024) characterised the broad spectrum of action of these compounds, but were limited to laboratory tests without field verification. T. Tsotetsi *et al.* (2022) showed that *Bacillus* bacteria activate plant stress resistance mechanisms, but did not investigate the relationship between metabolite concentration and actual changes in damage. In contrast to these authors, this study combined microbiological, biochemical,

and phytopathological analyses, which tracked both antagonistic and biostimulating effects simultaneously. This approach proved the real effectiveness of *B. subtilis* and *B. amyloliquefaciens* strains in long-term control of pathogens and increasing the viability of crops in field conditions.

Within the framework of environmentally oriented microbiological studies, P. Vasantha-Srinivasan *et al.* (2025) and K.M. Figueroa-Brambila *et al.* (2023) showed that representatives of the *Bacillus* genus are effective not only against fungi but also against soil nematodes, thanks to their ability to synthesise enzymes that destroy the cuticle of parasites. However, P. Vasantha-Srinivasan *et al.* (2025) did not consider the associated effects on the rhizosphere microbiota, which limits the scope of the ecological balance after biotreatment. K.M. Figueroa-Brambila *et al.* (2023) investigated a new species, *Bacillus cabrialesii*, which exhibited high biocontrol activity, but the experiments were short-term and did not cover the dynamics of metabolite accumulation. In contrast to these results, the present study monitored the prolonged antagonistic action of *B. subtilis* and *B. amyloliquefaciens* over 90 days, with a quantitative assessment of the growth inhibition index (GII), which confirmed the consistency of the effect and the biocompatibility of the preparations with the soil microflora.

In the context of integrated approaches, A. Ortiz & E. Sansinenea (2023) and A. Fessia *et al.* (2022) addressed the genetic and physiological aspects of biocontrol. A. Ortiz & E. Sansinenea (2023) considered the possibility of creating genetically modified plants based on *Bacillus* genes, but did not provide practical evidence of their effectiveness in field conditions. A. Fessia *et al.* (2022) proved that the formation of *Bacillus* biofilms in the phyllosphere can enhance the antagonistic effect, but the mechanisms of biofilm stability remain unexplored. In contrast to these studies, the present study shows a real bioaggregation effect in the rhizosphere, confirmed by microscopic observations and a stable decrease in the plant damage index. This provides a practical advantage, as the results are based on field testing rather than laboratory models alone.

In the context of applied biotechnological research, S. Jang *et al.* (2023) and A. Ali *et al.* (2022) demonstrated that *Bacillus velezensis* GB03 has become an industrial standard among biostimulants due to the stability of its metabolites, while A. Ali *et al.* (2022) summarised a wide range of antagonistic properties of bacteria against fungal phytopathogens. However, S. Jang *et al.* (2023) did not evaluate the long-term effect on crop viability, and A. Ali *et al.* (2022) were limited to a description of the mechanisms of action without experimentally verifying the duration of biocontrol. Compared to these works, the presented study has greater empirical depth: a consistent increase in the antagonistic activity of *B. subtilis* and *B. amyloliquefaciens* over time has been confirmed, morphological changes in pathogens and the preservation of plant health throughout the growing season have been recorded. This makes the results more significant for practical implementation in biological crop protection systems.

As part of microbiological experiments, T. Maciag *et al.* (2023) and C.P. Serrão *et al.* (2024) examined the role of *Bacillus* spp. in the formation of multicomponent biocontrol systems. T. Maciag *et al.* (2023) found that synthetic consortia involving *Bacillus subtilis* and *Pseudomonas fluorescens* can improve phytoprotection through the mutual exchange of signalling metabolites, but the individual effectiveness of each species remained unclear. C.P. Serrão *et al.* (2024), based on a meta-analysis of a 22-year array of publications, emphasised that the potential of *Bacillus* for biocontrol increases when combined with natural stimulants, but noted the unpredictability of the effect in different climatic regions. The present study demonstrates that *B. subtilis* and *B. amyloliquefaciens* exhibit stable antagonistic activity without the need for synergy, confirming their reliability as independent bioagents.

In the context of agronomic and applied research, O.G. Marisel *et al.* (2024) and M.M.M. Abd-Elgawad (2024) addressed the potential of *Bacillus* as growth stimulants and bioprotective agents. O.G. Marisel *et al.* (2024) found that *Bacillus thuringiensis* B3 improved the development of lettuce and tomatoes due to its phosphate-mobilising ability, but did not analyse its effect on phytopathogens. M.M.M. Abd-Elgawad (2024) proved that *Bacillus* spp. effectively suppresses nematode infections, but did not investigate its interaction with fungal and bacterial agents. In contrast to these studies, the present work investigated the simultaneous effect of *B. subtilis* and

B. amyloliquefaciens on three main groups of pathogens – fungal, bacterial and soil – which demonstrated a broader spectrum of biocontrol activity.

In the context of ecological and technological research, S.R. Prabhukarthikeyan *et al.* (2022) and L.R. Sales & E.C. Rigobelo (2024) addressed the integration of *Bacillus* into sustainable farming strategies. S.R. Prabhukarthikeyan *et al.* (2022) showed that the use of *Bacillus* increased crop resistance to abiotic stresses, but the results did not include a quantitative measurement of pathogen inhibition. L.R. Sales & E.C. Rigobelo (2024), studying the role of *Bacillus sp.* in reducing chemical loads, confirmed the ability of these bacteria to reduce the need for fungicides by 30-40%, but did not track the dynamics of action during the growing season. In this study, the antagonistic effect of *B. subtilis* and *B. amyloliquefaciens* was tested over a period of 6 to 72 hours, confirming the stability of the bioprotective effect in field conditions, which gives the results greater applied reliability. Thus, the results obtained proved that the use of *Bacillus subtilis* and *Bacillus amyloliquefaciens* effectively suppressed the development of major phytopathogens, increased the proportion of healthy plants, and ensured a sustained improvement in biometric and microbiological indicators throughout the growing season.

CONCLUSIONS

During the study, a comprehensive assessment of the antagonistic activity of *Bacillus subtilis* and *Bacillus amyloliquefaciens* strains against the main phytopathogens – *Fusarium oxysporum*, *Alternaria solani* and *Pseudomonas syringae* – was conducted, and their effect on plant growth and soil microflora was analysed. A comparative analysis of time points showed that *B. subtilis* consistently exceeded *B. amyloliquefaciens* in inhibitory capacity: the average diameters of the inhibition zones were 10-15% larger, and the growth inhibition index (GII) was 5-8% higher. This indicates higher biosynthetic activity of *B. subtilis*, in particular in the production of lysozyme-type enzymes (β-glucanases, chitinases, proteases) that destroy the cell walls of fungi and bacteria. In the control samples, the pathogens retained a stable morphology, confirming that the antagonism was caused by the cultural metabolites of *Bacillus*. Seasonal data showed a clear advantage of *B. subtilis* in reducing the damage index: for *F. oxysporum*, by 33.0 percentage points (from 53.8% to 20.8%), for *A. solani*, by 29.0 percentage points, and for *P. syringae*, by 27.1 percentage points. The proportion of healthy plants increased from 51.5-56.3% in the control to 86.2-87.3% with *B. subtilis* and 82.4-84.1% with *B. amyloliquefaciens*. These results indicate a sustained biocontrol effect that persisted throughout the growing season. Biometric parameters (height, number of leaves, root weight) increased by 20-40% compared to the control, which exceeded the effectiveness of the biological standard by 5-10%. All studies showed no toxicity, biological stress or soil degradation. Biological preparations maintained the balance of saprophytes (1.8×10^6 CFU/g), actinomycetes (1.9×10^6 CFU/g) and fungi (1.8×10^5 CFU/g), and the enzymatic activity of dehydrogenases reached 103% of the control. The limitation of the study is that the experiments were conducted only within one soil type and without analysing the influence of climatic factors. Further research should address the effectiveness of *Bacillus subtilis* and *Bacillus amyloliquefaciens* in different agroecological zones and in combination with other microbial consortia.

ACKNOWLEDGEMENTS

None.

FUNDING

None.

CONFLICT OF INTEREST

None.

REFERENCES

[1] Abd-Elgawad, M.M.M. (2024). *Bacillus* species: Prospects and applications for the root-knot disease management. In *Microbial biostimulants* (pp. 79-105). New York: Apple Academic Press.

[2] Ajuna, H.B., Lim, H.-I., Moon, J.-H., Won, S.-J., Choub, V., Choi, S.-I., Yun, J.-Y., & Ahn, Y.S. (2024). The prospect of antimicrobial peptides from Bacillus species with biological control potential against insect pests and diseases of economic importance in agriculture, forestry and fruit tree production. *Biotechnology & Biotechnological Equipment*, 38(1), article number 2312115. doi: 10.1080/13102818.2024.2312115.

[3] Ali, A., *et al*. (2022). Antagonistic potential of bacterial species against fungal plant pathogens (FPP) and their role in plant growth promotion (PGP): A review. *Phyton*, 91(9), 1859-1877. doi: 10.32604/phyton.2022.021734.

[4] Ayaz, M., *et al*. (2023). Bacterial and fungal biocontrol agents for plant disease protection: Journey from lab to field, current status, challenges, and global perspectives. *Molecules*, 28(18), article number 6735. doi: 10.3390/molecules28186735.

[5] Blanco Crivelli, X., Cundon, C., Bonino, M.P., Sanin, M.S., & Bentancor, A. (2024). The complex and changing genus *Bacillus*: A diverse bacterial powerhouse for many applications. *Bacteria*, 3(3), 256-270. doi: 10.3390/bacteria3030017.

[6] Boulahouat, S., Cherif-Silini, H., Silini, A., Bouket, A.C., Luptakova, L., Alenezi, F.N., & Belbahri, L. (2023). Biocontrol efficiency of rhizospheric *Bacillus* against the plant pathogen *Fusarium oxysporum*: A promising approach for sustainable agriculture. *Microbiology Research*, 14(3), 892-908. doi: 10.3390/microbiolres14030062.

[7] Convention on Biological Diversity. (1992, June). Retrieved from https://zakon.rada.gov.ua/laws/show/995_030#Text.

[8] Dutilloy, E., Oni, F.E., Esmaeel, Q., Clément, C., & Barka, E.A. (2022). Plant beneficial bacteria as bioprotectants against wheat and barley diseases. *Journal of Fungi*, 8(6), article number 632. doi: 10.3390/jof8060632.

[9] El-Saadony, M.T., *et al*. (2022). Plant growth-promoting microorganisms as biocontrol agents of plant diseases: Mechanisms, challenges and future perspectives. *Frontiers in Plant Science*, 13, article number 923880. doi: 10.3389/fpls.2022.923880.

[10] Fessia, A., Barra, P., Barros, G., & Nesci, A. (2022). Could *Bacillus* biofilms enhance the effectivity of biocontrol strategies in the phyllosphere? *Journal of Applied Microbiology*, 133(4), 2148-2166. doi: 10.1111/jam.15596.

[11] Figueroa-Brambila, K.M., Escalante-Beltrán, A., Montoya-Martínez, A.C., Díaz-Rodríguez, A.M., López-Montoya, N.D., Parra-Cota, F.I., & de Los Santos-Villalobos, S. (2023). *Bacillus cabrialesii*: Five years of research on a novel species of biological control and plant growth-promoting bacteria. *Plants*, 12(13), article number 2419. doi: 10.3390/plants12132419.

[12] Jang, S., Choi, S.K., Zhang, H., Zhang, S., Ryu, C.M., & Kloepper, J.W. (2023). History of a model plant growth-promoting rhizobacterium, *Bacillus velezensis* GB03: From isolation to commercialization. *Frontiers in Plant Science*, 14, article number 1279896. doi: 10.3389/fpls.2023.1279896.

[13] Khan, A.R., Mustafa, A., Hyder, S., Valipour, M., Rizvi, Z.F., Gondal, A.S., Yousuf, Z., Iqbal, R., & Daraz, U. (2022). *Bacillus* spp. as bioagents: Uses and application for sustainable agriculture. *Biology*, 11(12), article number 1763. doi: 10.3390/biology11121763.

[14] Kolchyk, O.V., Borovuk, I.V., Buzun, A.I., Illarionova, & Zazharska, N.M. (2024). Microorganisms' growth inhibition in poultry meat using *Bacillus* spp. *World's Veterinary Journal*, 14(3), 424-434.

[15] Kolomiiets, Y., Butsenko, L., Yemets, A., & Blume, Y. (2024). The use of PGPB-based bioformulations to control bacterial diseases of vegetable crops in Ukraine. *The Open Agriculture Journal*, 18(1), article number e18743315283724. doi: 10.2174/0118743315283724231220104524.

[16] Maciag, T., Kozieł, E., Rusin, P., Otulak-Kozieł, K., Jafra, S., & Czajkowski, R. (2023). Microbial consortia for plant protection against diseases: More than the sum of its parts. *International Journal of Molecular Sciences*, 24(15), article number 12227. doi: 10.3390/ijms241512227.

[17] Mahapatra, S., Yadav, R., & Ramakrishna, W. (2022). *Bacillus subtilis* impact on plant growth, soil health and environment: Dr. Jekyll and Mr. Hyde. *Journal of Applied Microbiology*, 132(5), 3543-3562. doi: 10.1111/jam.15480.

[18] Marisel, O.G., Yoania, R.R., Santiago, R.R., Alberto, Z.L.H., & Fernando, C.D.I. (2024). Bioprospecting a mountain-derived phosphorus-solubilizing bacterium: *Bacillus thuringiensis* B3 as a plant-growth promoter in lettuce and tomato horticultural crops. *Scientia Horticulturae*, 337, article number 113568. doi: 10.1016/j.scienta.2024.113568.

[19] Muthukumar, A., Raj, T.S., Prabhukarthikeyan, S.R., Kumar, R.N., & Keerthana, U. (2022). *Pseudomonas* and *Bacillus*: A biological tool for crop protection. In *New and future developments in microbial biotechnology and bioengineering* (pp. 145-158). Amsterdam: Elsevier. doi: 10.1016/B978-0-323-85577-8.00006-8.

[20] Ngalimat, M.S., Mohd Hata, E., Zulperi, D., Ismail, S.I., Ismail, M.R., Mohd Zainudin, N.A.I., Saidi, N.B., & Yusof, M.T. (2021). Plant growth-promoting bacteria as an emerging tool to manage bacterial rice pathogens. *Microorganisms*, 9(4), article number 682. doi: 10.3390/microorganisms9040682.

[21] Ortiz, A., & Sansinenea, E. (2022). The role of beneficial microorganisms in soil quality and plant health. *Sustainability*, 14(9), article number 5358. doi: 10.3390/su14095358.

[22] Ortiz, A., & Sansinenea, E. (2023). Genetically modified plants based on *Bacillus* genes and commercial *Bacillus*-based biopesticides for sustainable agriculture. *Horticulturae*, 9(9), article number 963. doi: 10.3390/horticulturae9090963.

[23] Phillips, J.M., & Hayman, D.S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions British Mycological Society*, 55(1).

[24] Prabhukarthikeyan, S.R., *et al*. (2022). *Bacillus* rhizobacteria: A versatile biostimulant for sustainable agriculture. In *New and future developments in microbial biotechnology and bioengineering* (pp. 33-44). Amsterdam: Elsevier. doi: 10.1016/B978-0-323-85163-3.00009-0.

[25] Reshetnikov, M.V., & Patyka, V.P. (2023). Bacteria-antagonists of the agents of soryz bacterial diseases. *Agricultural Science and Practice*, 10(3), 46-60. doi: 10.15407/agrisp10.03.046.

[26] Sales, L.R., & Rigobelo, E.C. (2024). The role of *Bacillus* sp. in reducing chemical inputs for sustainable crop production. *Agronomy*, 14(11), article number 2723. doi: 10.3390/agronomy14112723.

[27] Serrão, C.P., Ortega, J.C.G., Rodrigues, P.C., & de Souza, C.R.B. (2024). *Bacillus* species as tools for biocontrol of plant diseases: A meta-analysis of twenty-two years of research, 2000-2021. *World Journal of Microbiology and Biotechnology*, 40(4), article number 110. doi: 10.1007/s11274-024-03935-x.

[28] Toma, R.C., Boiu-Sicuia, O.A., Diguță, F.C., Ciucă, M., Matei, F., & Cornea, C.P. (2023). Selected plant protection *Bacillus* strains increase food safeness by inhibiting human pathogenic bacteria. *Romanian Agricultural Research*, 40, 609-619. doi: 10.59665/rar4057.

[29] Tsotetsi, T., Nephali, L., Malebe, M., & Tugizimana, F. (2022). *Bacillus* for plant growth promotion and stress resilience: What have we learned? *Plants*, 11(19), article number 2482. doi: 10.3390/plants11192482.

[30] Vasantha-Srinivasan, P., Park, K.B., Kim, K.Y., Jung, W.J., & Han, Y.S. (2025). The role of *Bacillus* species in the management of plant-parasitic nematodes. *Frontiers in Microbiology*, 15, article number 1510036. doi: 10.3389/fmicb.2024.1510036.

[31] Vasques, N.C., Nogueira, M.A., & Hungria, M. (2024). Increasing application of multifunctional *Bacillus* for biocontrol of pests and diseases and plant growth promotion: Lessons from Brazil. *Agronomy*, 14(8), article number 1654. doi: 10.3390/agronomy14081654.

[32] Wang, H., Liu, R., You, M.P., Barbetti, M.J., & Chen, Y. (2021). Pathogen biocontrol using plant growth-promoting bacteria (PGPR): Role of bacterial diversity. *Microorganisms*, 9(9), article number 1988. doi: 10.3390/microorganisms9091988.

Бактерії роду *Bacillus* як ефективний та безпечний засіб захисту рослин від патогенних мікроорганізмів

Софія Козлова

Аспірант, молодший науковий співробітник
Український інститут експертизи сортів рослин
03041, вул. Горіхуватський шлях, 15, м. Київ, Україна
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
https://orcid.org/0009-0000-5532-6866

Анотація. Метою дослідження було встановити взаємозв'язок між антагоністичною активністю штамів *Bacillus* та їх впливом на ріст рослин і мікробіологічну рівновагу ґрунту. У роботі застосовували комплекс мікробіологічних, фізіологічних і статистичних методів, зокрема метод агарових лунок для визначення антагонізму, метод серійних розведень для підрахунку колоній, трифенілтетразолій-хлоридний метод для оцінки активності дегідрогеназ і дисперсійний аналіз (ANOVA) для перевірки достовірності результатів. Показано, що *B. subtilis* стабільно перевищував *B. amyloliquefaciens* за інгібувальною здатністю: середні діаметри зон пригнічення були більшими на 10-15 %, а індекс пригнічення росту – на 5-8 %. Для *Fusarium oxysporum* зниження індексу ураження становило 33,0 процентних пункти (з 53,8 % до 20,8 %), для *Alternaria solani* – 29,0 процентних пунктів для *Pseudomonas syringae* – 27,1 процентних пункта. Частка здорових рослин збільшувалася з 51,5-56,3 % у контролі до 86,2-87,3 % при застосуванні *B. subtilis* і 82,4-84,1 % при *B. amyloliquefaciens*. Біопрепарати стимулювали ріст рослин: висота, кількість листків і маса кореня зростали на 20-40 % порівняно з контролем, перевищуючи ефективність біологічного стандарту на 5-10 %. Протягом експерименту не виявлено жодних ознак фітотоксичності чи деградації ґрунту. Біопрепарати підтримували стабільну чисельність сапрофітів ($1,8 \times 10^6$ КУО/г), актиноміцетів ($1,9 \times 10^6$ КУО/г) і грибів ($1,8 \times 10^5$ КУО/г), а активність дегідрогеназ досягала 103 % від контролю. Отримані результати підтвердили, що *Bacillus subtilis* і *Bacillus amyloliquefaciens* є ефективними антагоністами фітопатогенів і безпечними біостимуляторами, здатними забезпечити підвищення продуктивності та стабілізацію мікробіоценозу ґрунту. Їх застосування рекомендовано як екологічно стійку альтернативу хімічним фунгіцидам у системах сталого землеробства

Ключові слова: антагонізм; біоконтрольна дія; індекс ураження; екологічна безпека; ризосфера

БІОЛОГІЧНІ СИСТЕМИ: Теорія та Інновації

Науковий журнал
Том 16, № 4. 2025

Заснований у 2010 р. Періодичність випуску: щоквартально

Оригінал-макет видання виготовлено у відділі науково-технічної інформації
Національного університету біоресурсів і природокористування України

Відповідальний редактор:
О. Наумовська

Підписано до друку 27 листопада 2025 р.
Формат 70*100/16
Умов. друк. арк. 9,6
Наклад 100 прим.

Адреса редакції:
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 13, м. Київ, Україна
Тел.: +38(044)-258-42-63
E-mail: bs@bioscience.com.ua
https://bioscience.com.ua/uk

BIOLOGICAL SYSTEMS: Theory and Innovation

Scientific Journal
Volume 16, No. 4. 2025

Founded in 2010. Frequency: quarterly

The original layout of the publication is made in the Department of Scientific and Technical Information of National University of Life and Environmental Sciences of Ukraine

Managing Editor:
O. Naumovska

Signed for print of November 27, 2025
Format 70*100/16
Conventional printed pages 9.6
Circulation 100 copies

Editors office address:
National University of Life and Environmental Sciences of Ukraine
03041, 13 Heroiv Oborony Str., Kyiv, Ukraine
Tel.: +38(044)-258-42-63
E-mail: bs@bioscience.com.ua
https://bioscience.com.ua/en