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Original Research

Macrophyte Diversity and Ecosystem Services in Wetlands of Eastern Ranchi: A Multi-site Analysis of Abundance, Frequency, and Importance Value Index Patterns

Jitendra Mahato [1], Sujit Ghosh [2*]

Abstract

Macrophytes, visible aquatic and semi-aquatic plants, are integral components of wetland ecosystems, playing crucial roles in maintaining biodiversity, providing ecosystem services, and ensuring ecological stability. This research examines the distribution, abundance, frequency, diversity, and ecosystem services of macrophytes in several wetlands (W1-W7) located in the eastern part of Ranchi district, Jharkhand, India. The study sites include Bundu Lake, Hindalco Pond, Choga Bada Talab, Raja Bandh, Kita Uparbandh, Tamar Bada Talab, and Rukka Dam. A quantitative assessment using quadrat sampling and phytosociological methods was conducted from September 2022 to March 2023 to ensure accurate data collection and analysis. The study documented 78 macrophyte species belonging to 33 families and 58 genera. Emergent macrophytes were found to be the dominant life form, constituting 69% of the recorded species. Several species demonstrated high frequency and abundance across multiple study sites, including *Pontederia crassipes* Mart., *Alternanthera philoxeroides* (Mart) Griseb., *Hydrilla verticillata* (L.f.) Royle, *Ipomoega aquatica* Forssk., and *Nymphoides hydrophyllum* (Lour.) kuntze. To assess the diversity and ecological characteristics of the macrophyte communities, various diversity indices were calculated. These included the Shannon-Wiener index, Simpson's index, Margalef index, and Pielou's Evenness index. The results of these analyses revealed high levels of macrophyte diversity and evenness within the studied wetlands. Twenty-one macrophytes were identified as key species and nine species as rare species based on their IVI (Importance Value Index) scores. The key species were found to provide essential ecosystem services, including erosion control, water quality improvement, nutrient cycling, carbon sequestration, and habitat provision for other organisms. This research contributes significantly to our understanding of macrophyte diversity patterns and traits-based ecosystem services in wetland

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ecosystems. The findings of this study have important implications for the development and implementation of effective conservation strategies for these vital wetland ecosystems in the eastern Ranchi (W1- W7).

Keywords

Ecosystem services, key species, macrophytes, quantitative assessment, wetland

Raznolikost makrofitov in ekološke funkcije v mokriščih vzhodnega Ranchija: Analiza vzorcev številčnosti, pogostosti in indeksa vrednosti pomembnosti na več lokacijah

Izvleček

Makrofitiso sestavni del mokrišćnih ekosistemov, saj imajo ključno vlogo pri ohranjanju biotske raznovrstnosti, zagotavljanju ekosistemskih storitev in zagotavljanju ekološke stabilnosti. Ta raziskava preučuje razširjenost, številčnost, pogostost, raznolikost in ekološko funkcijo makrofitov v več mokriščih (W1-W7), ki se nahajajo v vzhodnem delu okrožja Ranchi v Džarkhandu v Indiji. Študijska območja vključujejo jezero Bundu, ribnik Hindalco, Choga Bada Talab, Raja Bandh, Kita Uparbandh, Tamar Bada Talab in jez Rukka. Kvantitativna ocena z uporabo kvadratnega vzorčenja in fitosocioloških metod je potekala od septembra 2022 do marca 2023, s čimer smo želeli zagotovitinatančnost zbranih podatkov in analiz. V študiji je bilo dokumentiranih 78 vrst makrofitov, ki pripadajo 33 družinam in 58 rodovom. Ugotovljeno je bilo, da so prevladujoča življenjska oblika emergentni makrofiti, ki so predstavljali 69 % zabeleženih vrst. Več vrst je pokazalo visoko pogostost in številčnost na več študijskih območjih, vključno s *Pontederia crassipes* Mart, *Alternanthera philoxeroides* (Mart) Griseb, *Hydrilla verticillata* (L.f.) Royle, *Ipomoea aquatica* Forssk in *Nymphoides hydrophyllum* (Lour.) kuntze. Za oceno raznolikosti in ekoloških značilnosti združb makrofitov so bili izračunani različni indeksi raznolikosti. Med njimi so bili Shannon-Wienerjev indeks, Simpsonov indeks, Margalefov indeks in Pieloujev indeks enakomernosti. Rezultati teh analiz so pokazali visoko stopnjo raznolikosti in enakomernosti makrofitov v preučevanih mokriščih. Enaindvajset makrofitov je bilo opredeljenih kot ključne vrste, devet vrst pa kot redke vrste na podlagi njihovih ocen IVI (Importance Value Index). Ugotovljeno je bilo, da ključne vrste zagotavljajo bistvene ekosistemske storitve, vključno z nadzorom erozije, izboljšanjem kakovosti vode, kroženjem hranil, sekvestracijo ogljika in zagotavljanjem habitatov za druge organizme. Ta raziskava pomembno prispeva k razumevanju vzorcev raznolikosti makrofitov in ekosistemskih storitev, ki temeljijo na lastnostih, v mokrotnih ekosistemih. Ugotovitve te študije imajo pomembne posledice za razvoj in izvajanje učinkovitih strategij ohranjanja teh ključnih mokrišćnih ekosistemov v vzhodnem Ranchiju (W1- W7).

Ključne besede

ekološka vloga, ključne vrste, makrofiti, kvantitativna ocena, mokrišče

Introduction

Wetlands are the most vital ecosystems on our planet, offering a wide range of ecological services such as water purification, flood control, carbon storage, and the conservation of biodiversity. These ecosystems help regulate water cycles and are rich sources of biodiversity, supporting numerous species, genetic variations, and diverse ecosystems (Mitsch et al., 2015; Shiji et al., 2016). Among the key players in these environments are macrophytes, large aquatic plants that you can see with the naked eye. Macrophytes contribute significantly to the health of aquatic ecosystems by promoting sediment deposition, carbon capture, water filtration, and the removal of pollutants. Additionally, they serve as sources of bioenergy biochar and help mitigate the impacts of climate change.

Beyond these environmental roles, macrophytes provide natural medicines, fibres, and other resources that benefit human society, reinforcing the link between biodiversity and human well-being (Kumar et al., 2023). Unfortunately, these vital plants are increasingly under threat. Pollution, habitat destruction, invasive species, and climate change are all taking a toll on macrophyte populations, leading to degraded ecosystems and a loss of biodiversity (Lesiv et al., 2020). Human activities, such as unsustainable water management, overharvesting, sediment build-up, and the use of chemicals, further compound these challenges, damaging both macrophytes and the essential services they provide.

In the eastern part of Ranchi district, Jharkhand, India, several wetlands serve as important habitats for a variety of macrophyte species. Wetlands like Bundu Lake, Hindalco Pond, Choga Bada Talab, Raja Bandh, Kita Uparbandh, Tamar Bada Talab, and Rukka Dam host a diverse range of aquatic vegetation. These plants play a crucial role in maintaining the health and functioning of these ecosystems. Understanding how these macrophytes are distributed, how abundant they are, how frequently they appear, and what services they offer to the ecosystem is essential if we are to develop effective conservation and management strategies. Various studies have looked at macrophyte diversity and the ecological significance of wetlands in other regions (Swamy et al., 2016; Xu et al., 2019; Bhanja et al., 2023). In the Chota Nagpur Plateau, where Ranchi district is located, researchers have explored macrophyte diversity in places like Ranchi Lake and Kanke Dam. For instance, Lal and Lal (2021) found 36 macrophyte species in Kanke Dam and 17 species in Ranchi Lake. Other studies have mentioned the macrophyte diversity in various wetlands across the Ranchi district in their broader floristic surveys (Verma and Pandeya, 2008; Mukherjee and Kumar, 2020). However, most of these studies focus on individual sites and don't provide a broader picture of species distribution, abundance, or diversity across multiple wetlands. Although macrophytes are well known for their role in delivering ecosystem services (Thomaz, 2023), there is a lack of site-specific information in the wetlands of the eastern Ranchi region that connects species composition to ecosystem functions. Furthermore, the lack of precise suggestions based on in-depth ecological data in the region's current conservation strategies for wetlands impairs the development of efficient management strategies.

Comprehensive research on the distribution, abundance, frequency, diversity, and ecosystem services of macrophytes in the seven selected wetlands in the eastern portion of Ranchi district is missing despite the ecological relevance of wetlands and the value of these plants. The lack of comparative study of species frequency and diversity indices, the scarcity of data on specific species distributions and abundances, and the lack of knowledge on ecosystem services are some of the major research gaps. To fill these gaps, a thorough investigation of the biological patterns and ecosystem services provided by the macrophyte species in these wetlands is necessary. The primary goal of this research is to explore the diversity of macrophytes, their abundance and distribution patterns, and the ecosystem services they provide across seven selected wetlands in the eastern Ranchi district.

Materials and Methods

The study area

The study was carried out in Ranchi District, the capital of Jharkhand, India, which is located between 85°15′-85°23′ E longitude and 23°21′-23°87′ N latitude, spanning 7,698 square kilometres of steep terrain. The district is located in the southern section of the Chota Nagpur Plateau, an extension of the Deccan Plateau, and is bordered to the north by Ramgarh, Hazaribagh, and Chatra, to the south by Khunti, to the west by Latehar, Lohardaga, and Gumla, and to the east by Saraikela and Purulia (West Bengal). Ranchi has a humid subtropical climate, with summer temperatures ranging from 20-40°C and winter temperatures ranging from 1-25°C, and it receives both seasonal and cyclonic rainfall (Rani et al., 2011). The Subarnarekha and its tributaries form the principal river system.

Selection of Wetlands

For the quantitative analysis of wetland biodiversity and ecological conditions, seven wetlands from the eastern region of the district were selected, each representing different blocks. The wetlands include Bundu Lake (W1), Hindalco Pond (W2), Choga Bada Talab (W3), Raja Bandh (W4), Kita Upar Bandh (W5), Tamar Bada Talab (W6), and Rukka Dam (W7) (Fig. 1). Table 1 provides general information about these selected wetlands, including their geographical coordinates, elevation, surface area, and macrophyte cover. The selection of these wetlands aimed to capture

both urban and rural settings, allowing for a comparative analysis of human influence and ecological health across varying landscapes.

The catchment areas and corresponding human influences on these wetlands were assessed to understand their ecological status. W1 (Bundu Lake), an urban wetland covering 40.47 hectares, is located near markets, human settlements, and agricultural land. It experiences heavy pollution due to urban sewage, domestic waste, agricultural runoff, and shrinkage caused by human settlement expansion, road adjacency, and recreational use. Similarly, W2 (Hindalco Pond), a smaller 2.43-hectare urban wetland near Muri Station, is significantly impacted by industrial activities and urban settlements. This wetland faces severe pollution from industrial and domestic sewage, siltation, and industrial encroachment. In contrast, W3 (Choga Bada Talab), a rural wetland spanning 10.93 hectares, is surrounded by agricultural land and open grasslands. Its pollution mainly stems from agricultural runoff, domestic use, and irrigation, but it is less affected by human interference compared to the urban wetlands. Another rural wetland, W4 (Raja Bandh), covering 9.41 hectares, has a catchment area consisting of hilly uplands, agricultural fields, and open grasslands. Pollution sources in this wetland include road construction, domestic activities, cattle bathing, and the partial conversion of land for agriculture. W5 (Kita Uparbandh), an urban wetland of 2.02 hectares, is bordered by natural hilly vegetation, agricultural land, and a major road. It suffers from pollution caused by domestic sewage, proximity to human settlements, road adjacency, and shrinkage due to increasing urbanization. W6 (Tamar Bada Talab), a rural wetland of 5.67 hectares, is less affected by human activities, with catchment areas consisting of open grassland and agricultural land. Pollution in this wetland is primarily due to agricultural runoff and limited domestic use, making it one of the least impacted wetlands in the study.

Lastly, W7 (Rukka Dam) is a riverine, natural wetland with minimal human influence, spanning a large rural area (10199.77 hectares). Its catchment areas include agricultural land, open grasslands, and forested regions, with no major anthropogenic disturbances. Pollution in W7 is limited to agricultural runoff and occasional recreational activities, maintaining its status as a relatively pristine ecosystem. The variety of settings across these seven wetlands allows for a comprehensive analysis of how human activities influence wetland biodiversity and ecological conditions.

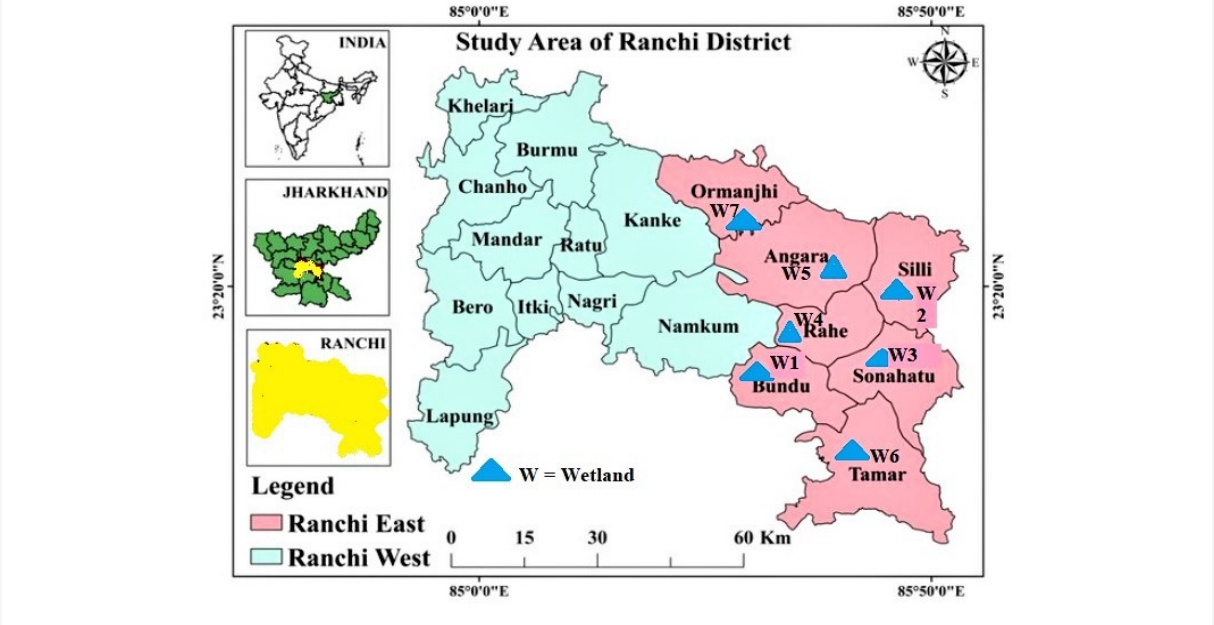

Figure 1. Location map of the study area with designated sampling sites (W1-W7) (Source: Mishra and Lal, 2023 and modified based on the location of wetlands).

Slika 1. Lokacijska karta območja študije z označenimi mesti vzorčenja (W1-W7) (Vir: Mishra in Lal, 2023 in spremenjeno na podlagi lokacije mokrišč).

Table 1. Overview of the selected wetlands.

Tabela 1. Pregled izbranih mokrišč.

Name of the Wetlands, (Blocks).	Latitude	Longitude	Altitude	Size hectares	Existing Sp.
Bundu Lake. (Bundu).	23.1614 N	85.586489 E	259.47 met.	40.47 ha.	31
Hindalco Pond. Muri, (Silli).	23.38126 N	85.867212 E	198.63 met.	2.43 ha.	25
Choga Bada Talab. Choga, (Sonahatu).	23.21299 N	85.7433049 E	190.09 met.	10.93 ha.	27
Raja Bandh. Rahe, (Rahe).	23.24986 N	85.6375088 E	251.66 met.	9.41 ha.	27
Kita Uparbandh. Kita, (Angara).	23.36492 N	85.765632 E	251.55 met.	2.02 ha.	25
Tamar Bada Talab. Tamar, (Tamar).	23.05430 N	85.65870 E	189.46 met.	5.67 ha.	29
Rukka Dam, (Ormanjhi, only Rukka portion.)	23.43663 N	85.462674 E	596 met.	10199.77 ha.	39

Sampling methodology

Fieldwork was conducted from September 2022 to March 2023, spanning the post-monsoon to winter seasons. Quadrat sampling was used to assess macrophytes qualitatively and quantitatively. Ten 1 m × 1 m quadrats were randomly placed throughout each wetland, with three replicates per site. Sampling was carried out up to 2 m from the wetland's periphery to its centre. The macrophytes that occurred or fell within each sampling unit were counted by hand and listed by species (Margalef, 1969).

Identification and nomenclature

Plant species were identified using standard protocols, work and keys provided by the books of 'Aquatic and Wetland Plants of India' (Cook 1996) and 'Wetland Flora of West Bengal' (Chowdhury and Chowdhury 2022). Valid scientific names were cross-checked using the Plants of the World Online (POWO 2024) and World Flora Online (WFO 2024) databases. Voucher samples of identified macrophytes were prepared and deposited at the Botany Department herbarium of Sidho-Kanho-Birsha University (SKBU), Purulia, West Bengal, India, for future reference and verification.

Data analysis

The phytosociological characteristics were determined using established procedures (Margalef, 1969). The diversity indicators, including Shannon-Wiener, Simpson's, species richness, and species evenness, were calculated using Magurran's (2004) methods. The number of individuals in each species was counted to calculate phytosociological measures such as percent frequency, frequency class (Raunkiaer, 1934), relative frequency, abundance, density, relative density, and IVI (Importance Value Index) using the following formulas.

$$\text{Frequency (\%)} = \frac{\text{Total number of quadrants in which species has occurred}}{\text{Total number of quadrants studied}} \times 100$$

$$\text{Relative Frequency} = \frac{\text{Frequency of a particular species}}{\text{Sum of frequencies of all species}} \times 100$$

$$\text{Abundance} = \frac{\text{Total number of indivisuals of the species}}{\text{Total number of quadrants in which the species has occurred}}$$

$$\text{Relative Abundance} = \frac{\text{Abundance of a particular species}}{\text{Total abundance of all species}} \times 100$$

$$\text{Density} = \frac{\text{Total number of indivisuals of the species}}{\text{Total number of quadrants studied}}$$

$$\text{Relative Density} = \frac{\text{Density of a particular species}}{\text{Total density of all species}} \times 100$$

IVI = (Relative Frequency + Relative Abundance + Relative Density).

Statistical analyses

Microsoft Excel was used to conduct statistical analyses comparing macrophyte diversity and other factors among the seven wetlands (W1 to W7). The data was interpreted by the application of descriptive statistics and comparative analyses.

Results

Macrophyte Species Distribution and Abundance

Across the seven wetlands (W1 to W7), a total of 78 macrophyte species (Table 2) from 33 families and 58 genera were recorded. Emergent macrophytes dominated the flora, making up 69% of the total, followed by rooted floating-leaf species (12%), submerged species (11%), and free-floating species (8%).

Emergent macrophytes were the most prevalent, with 54 species recorded, including *Cyperus iria* L. particularly abundant in Wetland W5, *Paspalum distichum* L. in Wetlands W3 and W5, and *Echinochloa colonum* (L.) Link. in Wetlands W4, W5, and W7. Rooted with floating-leaved macrophytes were represented by nine species, with *Nymphaea pubescens* Willd., *Nymphoides hydrophyllum* (Lour.) kuntze, and *Trapa natans* var. *bispinosa* (Roxb.) Makino. being the most common. Free-floating macrophytes comprised six species, with *Lemna perpusilla* Torr. and *Spirodela polyrhiza* (L.) Schleid. frequently observed in Wetlands W1, W2, W6, and W7 (Table 2). Lastly, nine species of submerged macrophytes were documented, with *Potamogeton crispus* L. and *Potamogeton nodosus* Poir. dominating across the study sites.

Table 2. Distribution of macrophyte species with IVI and growth forms.

Tabela 2. Razporeditev vrst makrofitov z IVI in oblikami rasti.

Scientific Name	IVI W1	W2	W3	W4	W5	W6	W7	G.F.
Acmella paniculata (Wall ex DC.) R.K. Jansen	3.12	9.58			7.16		4.72	E
Actinoscirpus grossus (L.f.) Goetgh. & D.A. Simpson			7.01					E
Aeschynomene indica L.			7.36		4.17			E
Alternanthera philoxeroides (Mart) Griseb.	18.75	14.41			19.69	13.97	12.30	E
Alternanthera sessilis (L.) DC.	11.71	11.61		13.65	14.36	9.80	7.86	E
Ammannia baccifera L.				6.17				E
Azolla pinnata R.Br.	11.74	20.54				11.63	7.28	FF
Bacopa monnieri (L.) Wettst.			17.76					E
Centella asiatica (L.) Urb.					4.75		6.47	E
Ceratophyllum demersum L.						7.59		S
Ceratopteris thalictroides (L.) Brongn.					4.29		3.84	E
Chenopodium ficifolium Sm.		8.31						E
Colocasia esculenta (L.) Schott	3.86	7.16			5.01	4.54	2.87	E
Cyperus brevifolius (Rottb.) Hassk.							7.56	E
Cyperus compressus L.	10.40		14.52		12.55	13.25	10.75	E
Cyperus difformis L.	10.04			12.62	17.81	10.03		E
Cyperus flavidus Retz.							7.99	E
Cyperus fuscus L.							9.00	E,
Cyperus iria L.			14.25		17.94			E
Cyperus polystachyos Rottb.	5.98							E
Dentella repens (L.) J.R. Forst. & G. Forst.			13.42					E
Echinochloa colonum (L.) Link				15.10	16.24		11.18	E
Eclipta prostrata (L.) L.	7.03	9.39	7.66	9.29	9.44	7.68	6.05	E

Eleusine indica (L.) Geartn.						12.51	9.49	E
Evolvulus nummularius (L.) L.	7.75			12.62			5.13	E
Fimbristylis aestivalis (Retz.) Vahl	9.51							E
Fimbristylis alboviridis C.B. Clarke				12.95				E
Fimbristylis argentea (Rottb.) Vahl			15.62					E
Fimbristylis bisumbellata (Forssk.) Bubani	11.10		17.28	12.34				E
Fuirena ciliaris (L.) Roxb.	10.55				17.09	10.03	8.76	E
Grangea maderaspatana (L.) Poir.				8.67				E
Heliotropium indicum L.	4.39			9.26				E
Hydrilla verticillata (L.f.) Royle	18.15	15.88				18.29		S
Hygrophila auriculata (Schumach.) Heine			9.01					E
Ipomoea aquatica Forssk.	9.84	12.98	10.84	10.16	11.90	8.76	7.54	RFL
Ipomoea carnea Jacq.	6.69	6.68	7.01	9.26		5.46	3.10	E
Kyllinga melanosperma Nees	12.05							E
Lemna perpusilla Torr.	17.57	24.81				15.48	10.12	FF
Lindernia parviflora (Roxb.) Haines			11.77					E
Limnophila sessiliflora (Vahl) Blume					14.25			S
Ludwigia adscendens (L.) H. Hara			9.09			8.05	8.03	RFL
Ludwigia perennis L.	4.57		9.31	5.07	5.01	3.63		E
Louisiella paludosa (Roxb.) Landge				13.65				E
Marsilea minuta L.		18.99	15.42	11.03	16.34	14.62	8.16	RFL
Melilotus indicus (L.) All.		7.16						E
Nelumbo nucifera Geartn.	3.58		6.81			8.65		RFL
Nymphaea nouchali Burm.f.	3.69			3.67				RFL
Nymphaea pubescens Wild.	5.45	4.48	7.08	8.70	12.74	10.81		RFL
Nymphoides hydrophyllum (Lour.) Kuntze			22.84	20.23	12.39			RFL
Nymphoides indica (L.) Kuntze	12.80	p				13.43		RFL
Oldenlandia corymbosa L.			8.46	9.51	8.58	5.15		E
Ottelia alismoides (L.) Pers.		6.68						S
Panicum repens L.	12.95	21.07			17.32	13.53	10.83	E
Paspalum distichum L.			16.73	16.99	16.81	12.76		E
Persicaria barbata (L.) H. Hara							3.44	E
Persicaria hydropiper (L.) Delabre		7.16		10.81				E
Persicaria glabra (Willd.) M.Gomez			5.62				3.16	E
Phyla nodiflora (L.) Greene	9.16						5.79	E
Pistia stratiotes L.	10.22						15.15	FF
Polygonum plebeium R.Br.			14.52	12.62			7.28	E
Pontederia crassipes Mart.	20.72	21.91					17.20	FF

Pontederia vaginalis Burm.f.		6.68					4.18	E
Potamogeton crispus L.				16.15			9.13	S
Potamogeton nodosus poir.						11.28	9.24	S
Pouzolzia zeylanica (L.) Benn.		4.48						E
Ranunculus sceleratus L.		6.68						E
Rotala rotundifolia (Buch. -Ham. ex Roxb.) Koehne					17.07		9.97	E
Salvinia cucullata Bory							9.48	FF
Schoenoplectiella articulata (L.) Lye.			5.62					E
Schoenoplectiella senegalensis (Steud.) Lye				6.17			4.86	E
Scleromitrion brachypodum (DC.) T.C. Hsu				8.35				E
Spirodela polyrhiza (L.) Schleid.	17.20	23.26				16.44	8.84	FF
Torenia crustacea (L.) Chan & Schltdl.	9.08		7.35		7.88		6.71	E
Trapa natans var. *bispinosa* (Roxb.) Makino	6.51			15.98		14.56		RFL
Typha domingensis Pers.		9.58					4.58	E
Utricularia aurea Lour.				8.98		4.85		S
Utricularia stellaris L. f.	6.64	7.72	7.90		9.19	5.87	5.97	S
Vallisneria spiralis L.			9.75			7.34	6.00	S

(Abbreviations: G. F= Growth form, E = Emergent, S = Submerged, RFL = Rooted with floating leaves, FF = Free floating.)

Dominant families

To determine the dominant families across the study sites, a quantitative approach was employed. The proportion of species represented by each family was calculated by dividing the number of species within a particular family by the total number of species across all families. This proportion was then expressed as a percentage using the following formula:

$$\text{Percentage of Family} = \frac{\text{Number of Species in a Particular Family}}{\text{Total number of Species across all Families}} \times 100$$

The family with the highest percentage was identified as the dominant family, representing the largest contribution to the overall macrophyte diversity in the wetlands. This approach allowed for a clear assessment of the relative importance of each family in the macrophyte community. The Cyperaceae family was the most dominant based on species richness and percentage representation, with 16 species accounting for 20.51% of the total species.

Other notable families included Poaceae (6.41%), Araceae (5.12%), Polygonaceae (5.12%), Hydrocharitaceae (3.85%), Convolvulaceae (3.85%), Asteraceae (3.85%), and Rubiaceae (3.85%). These families made significant contributions to the overall macrophyte diversity in the wetlands (Fig. 2).

Frequency and Distribution of Macrophyte Species

The frequency of occurrence of various plant species across different wetlands varied considerably. Species such as *Pontederia crassipes* Mart. and *Alternanthera philoxeroides*, *Hydrilla verticillata* exhibited high frequency across various wetlands, suggesting adaptability to a range of environmental conditions. *Nymphoides hydrophyllum* showed a particularly high frequency in Wetland 3 (W3) and Wetland 4 (W4), with occurrences of 90% and 83.33%, respectively. Conversely, low-frequency species like *Acmella paniculata*, *Pouzolzia zeylanica* (L.) Benn., *Persicaria glabra* (Willd.) M. Gómezand *Torenia crustacea* (L.) Chan & Schltdl. were restricted to specific areas, likely due to their sensitivity to habitat modifications (see Table 3 for details).

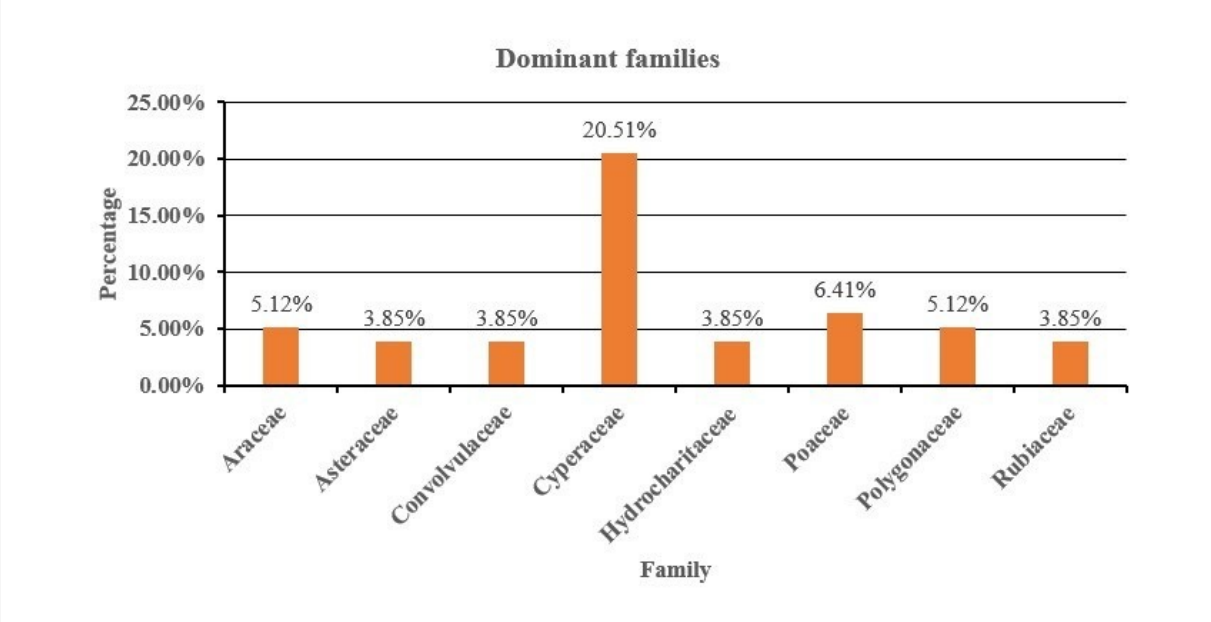

Figure 2. Distribution of dominant families across the study sites (W1-W7).

Slika 2. Porazdelitev dominantnih družin na raziskovalnih območjih (W1-W7).

Table 3. Frequency and distribution of macrophyte species.

Tabela 3. Pogostost in porazdelitev vrst makrofitov.

High Frequency Species	Frequency (%)	Wetlands	High Frequency Species	Frequency (%)	Wetlands
Pontederia crassipes	85.71%, 70%, 81.25%	W1, W2, W7	*Acmella paniculata*	14.29%	W1
Alternanthera philoxeroides	64.29%, 62.5%, 62.5%	W1, W5, W7	*Torenia crustacea*	14.29%, 20%	W1, W2
Alternanthera sessilis	50%, 50%	W1, W5	*Chenopodium ficifolium*	10%	W2
Hydrilla verticillata	57.14%, 70%	W1, W6	*Pouzolzia zeylanica*	10%	W2
Ipomoea aquatica	50%, 50%, 43.75%	W2, W3, W7	*Bacopa monnieri*	20%	W3
Nymphaea pubescens	60%, 62.5%	W6, W5	*Persicaria glabra*	20%, 18.75%	W3, W7
Panicum repens	70%	W2	*Persicaria hydropiper*	16.67%, 20%	W4, W2
Pistia stratiotes	81.5%	W7	*Ammannia baccifera*	16.67%	W4
Trapa natans var. bispinosa	60%, 58.33%	W6, W4	*Fimbristylis alboviridis*	16.67%	W4
Ludwigia adscendens	62.5%, 50%, 50%	W7, W3, W6	*Grangea maderaspatana*	16.67%	W4
Nymphoides hydrophyllum	90%, 83.33%	W3, W4	*Aeschynomene indica*	12.5%	W5
Nymphoides indica	50%, 50%	W2, W6	*Centella asiatica*	12.5%	W5
Potamogeton crispus	66.67%	W4	*Ceratopteris thalictroides*	12.5%, 25%	W7, W5
Salvinia cucullata	50%	W7	*Colocasia esculenta*	18.75%, 20%	W7, W6
Cyperus difformis	50%	W5	*Azolla pinnata*	10%	W6
			Lemna perpusilla	10%	W6
			Spirodela polyrhiza	10%	W6

Raunkiaer's Frequency Analysis

The frequency distribution of plant species within each wetland was calculated using Raunkaier's law of frequency classes (1934). The results are presented in Table 4. By analyzing and comparing them to Raunkaier's law of frequency classes (A > B > C ≥ D < E, where the value of the frequency class comprises class A= 53%, B=14%, C=9%, D= 8% and E=16%) across all wetlands (W1 - W7), significant insights were gained into the spatial distribution and diversity of macrophytes. This analysis highlights the varying patterns of species diversity, their spatial distribution, and the ecological status of these wetlands.

Wetland W1 had the highest percentage in Class B (64.52%), indicating a heterogeneous vegetation structure, while Wetland W2 displayed higher disturbance with lower frequencies in Classes C and E (4.00% and 0.00%, respectively). In Wetland W3, Class B dominated with a frequency of 55.55%, indicating moderate and stable diversity, while Class D was absent. Wetland W4 also showed a strong presence of Class B at 48.15%, with Classes A and C equally represented at 22.22%. Wetland W5 exhibited Class B at 48.00% and Class C at 36.00%, suggesting a less diverse community structure. In Wetland W6, Class B was again the most frequent at 44.83%, with limited diversity overall. Wetland W7 recorded Class B at 41.02% and Class A at 38.46%, reflecting a consistent distribution pattern across the wetlands. Hence, the study exposed distinct patterns of species diversity and ecological dynamics within these wetland ecosystems.

Diversity Indices and Evenness Analysis

The current study investigates macrophyte diversity and evenness across the wetlands under study (W1-W7) using several established biodiversity indices (Table 5), including the Shannon and Weiner diversity index (H'), Simpson's index of diversity (1-D), Margalef diversity index, and Pielou's evenness index (J'). These indices provide insights into the composition, richness, and evenness of macrophyte communities, offering a comprehensive view of their biodiversity status (Fig. 3).

Table 4. Raunkaier's frequency classes (%) of the study sites (W1-W7).

Tabela 4. Raunkaierjevi frekvenčni razredi (%) raziskovalnih območij (W1-W7).

Study sites	Class A	Class B	Class C	Class D	Class E	Raunkaier's Frequency
W1	9.68	64.52	19.36	3.22	3.22	A < B > C > D = E
W2	52.00	36.00	4.00	8.00	0.00	A > B > C < D > E
W3	22.22	55.55	18.53	0.00	3.70	A < B > C > D < E
W4	22.22	48.15	22.22	3.70	3.70	A < B > C > D = E
W5	8.00	48.00	36.00	8.00	0.00	A < B > C > D > E
W6	34.48	44.83	17.24	3.45	0.00	A < B > C > D > E
W7	38.46	41.02	10.26	5.13	5.13	A < B > C > D = E

Table 5. Values of different diversity Indices and evenness.

Tabela 5. Vrednosti različnih indeksov raznolikosti in enakomernosti.

Index	W1	W2	W3	W4	W5	W6	W7
Shannon and Weiner Diversity Index (H):	3.1903	2.9512	3.1383	3.1781	3.0264	3.2651	3.4968
Simpson's Index of Diversity (1- D):	0.9514	0.9367	0.9505	0.9545	0.9464	0.9542	0.9654
Margalef Diversity Index:	4.14533	3.853248	3.925082	3.887354	3.628268	4.207085	5.08069
Pielou's Evenness (J')	0.92904649	0.916832	0.952205	0.964278	0.940208	0.96965	0.954481

The Shannon-Weiner diversity index (H') revealed that Wetland W7 had the highest biodiversity (H' = 3.4968), indicating a well-diversified and evenly distributed species community. In contrast, Wetland W2 exhibited the lowest biodiversity (H' = 2.9512), suggesting reduced species richness and evenness. Simpson's Index of Diversity (1-D), which also considers species richness and evenness, showed high values for wetlands W1, W3, W4, W6, and W7, with scores ranging from 0.9505 to 0.9654. These high values indicate that these wetlands have diverse and stable ecosystems. However, W2 and W5 had slightly lower (1-D) values (0.9367 and 0.9464), consistent with their lower H' scores, reflecting reduced biodiversity or dominance by certain species.

The Margalef diversity index (DMg), which focuses on species richness, highlighted that W1 (4.14533), W3 (3.925082), W4 (3.887354), and W6 (4.207085) had relatively high species richness. Wetland W7 had the highest richness overall, while W5 exhibited the lowest species richness. The lower DMg values in W2 (3.853248) and W5 (3.628268) suggest that these wetlands experience reduced species richness, likely due to environmental stressors such as pollution and human activities. Pielou's Evenness index (J'), which measures species distribution and ecological balance, showed high evenness in wetlands W3, W4, W5, W6, and W7, indicating stable environmental conditions. Wetland W6 had the highest evenness score (0.96965), whereas W1 (0.92904) and W2 (0.91683) displayed the lowest evenness, possibly due to the influence of environmental or anthropogenic factors.

Categorization of IVI Values

The analysis of IVI values across these wetlands (W1-W7) revealed distinct patterns in the distribution of species based on their ecological significance. The important value index (IVI) serves as an indicator of a species' role within the community, considering its abundance, frequency, and dominance. Table 6 illustrates the categorization and ranges of IVI scores for each study site, highlighting species with low, intermediate, and high IVI values. The percentage of species with low, intermediate, and high IVI values varied among the wetlands, highlighting the diverse ecological roles and dominance of macrophyte species. Species with low IVI values, such as *Acmella paniculata*,

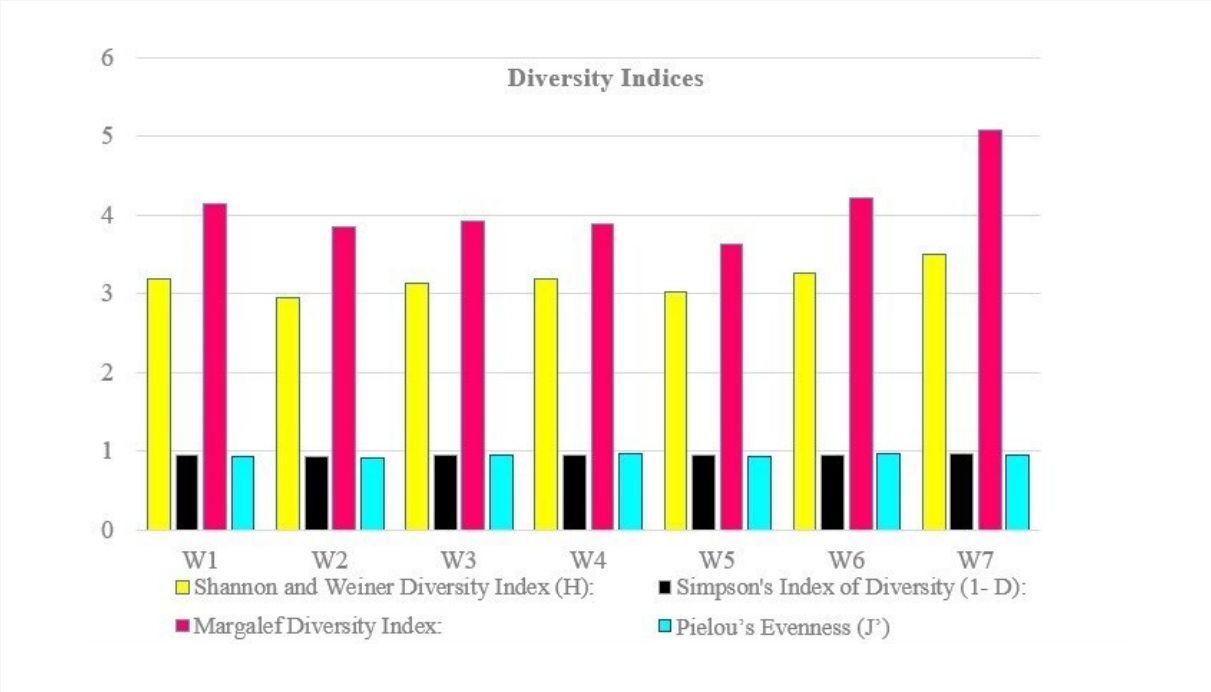

Figure 3. Comparative diversity indices of macrophytes at the study sites (W1-W7). (Shanon and Weiner, Simpson, Margalef, Pielou's index).

Slika 3. Primerjalni indeksi raznolikosti makrofitov na preučevanih območjih (W1-W7). (Shanon in Weiner, Simpson, Margalef, Pieloujev indeks)

Hydrilla verticillata, *Colocasia esculenta*, exhibit low abundance, frequency, or dominance across wetlands W1–W7, indicating their modest roles in wetland structure and functioning. Intermediate IVI species, which dominate most of the wetlands (W1–W7), make up 58–77% of the total macrophyte community. These species, such as *Alternanthera philoxeroides* and *Azolla pinnata*, contribute significantly to the stability of wetland ecosystems by maintaining biodiversity, habitat provisioning, and food supply for various organisms. High IVI species, like *Pontederia crassipes* and *Nymphoides hydrophyllum* are the most frequent and dominant, playing crucial roles in nutrient cycling, sediment retention, and habitat structuring.

Rare Species

Nine species were identified as rare based on their low relative density, frequency, dominance and importance value index (IVI). Significant rare species included *Grangea maderaspatana* (IVI 8.67) and *Scleromitrion brachypodum* (DC.) T.C. Hsu (IVI 8.35), both crucial for habitat provision and soil stabilization. These species were primarily found in W3 and W4 and are vulnerable to environmental changes and anthropogenic disturbances. Other rare species included *Actinoscirpus grossus* (IVI 7.01), *Schoenoplectiella articulata* (L.) Lye (IVI 5.62), *Ranunculus sceleratus* L. (IVI 6.68), *Ammannia baccifera* (IVI 6.16), *Ottelia alismoides* (L.) Pers. (IVI 6.68), *Ceratopteris thalictroides* (IVI 4.29) and *Pouzolzia zeylanica* (IVI 4.48).

Trait-Based Ecosystem Services of Macrophytes

In this study, 21 dominant macrophyte species were recognized as key species due to their high IVI, abundance, and density, contributing significantly to numerous ecosystem services (Table 7). These species include 52% emergent macrophytes, 10% submerged, 24% free-floating, and 14% rooted floating-leaved species.

Traits such as extensive root systems, dense root hairs, and rhizome formation are vital for soil stabilization, nutrient absorption, and sediment retention in wetlands. Species like *Alternanthera philoxeroides*, *Bacopa monnieri*, *Potamogeton crispus*, *Azolla pinnata*, *Lemna perpusilla* are particularly efficient at trapping sediments, preventing erosion and preserving the structural integrity of wetlands. Their rapid root growth, deep rooting systems and rhizome formation further enhance their ability to stabilize sediments and promote overall wetland health through significant above-ground biomass production. Dense root networks, fibrous roots and fine root hairs play a key role in retaining sediments, contributing significantly to the resilience and stability of wetland ecosystems.

In terms of water quality improvement, species such as *Hydrilla verticillata*, *Lemna perpusilla*, and *Spirodela polyrhiza* play key roles in nutrient uptake and pollutant absorption through root exudates. These species, along with *Nymphoides hydrophyllum*, *Pontederia crassipes* and *Rotala rotundifolia* contribute to sediment retention and

Table 6. Categorization and ranges of IVI scores of macrophytes in the study sites (W1-W7).

Tabela 6. Kategorizacija in razponi ocen IVI makrofitov na preučevanih območjih (W1-W7).

Study sites	Ranges of IVI	Low IVI Score	Intermediate IVI Score	High IVI Score
W1	3.12 - 20.72	*Acmella paniculata* (IVI-3.12)	*Azolla pinnata* (IVI- 11.74)	*Pontederia crassipes* (IVI- 20.72)
W2	4.48 - 24.81	*Nymphaea pubescens* (IVI 4.48)	*Alternanthera philoxeroides* (IVI 14.41)	*Lemna perpusilla* (IVI – 24.81)
W3	5.62 – 22.84	*Persicaria glabra* (IVI – 5.62)	*Cyperus iria* (IVI – 14.25)	*Nymphoides hydrophyllum* (IVI - 22.84)
W4	3.67 – 20.23	*Nymphaea nouchali* (IVI – 3.67)	*Fimbristylis bisumbellata* (IVI – 12.34)	*Nymphoides hydrophyllum* (IVI – 20.23)
W5	4.17 – 19.69	*Aeschynomene indica* (IVI – 4.17)	*Ipomoea aquatica* (IVI – 11.90)	*Alternanthera philoxeroides* (IVI – 19.69)
W6	3.63 – 18.29	*Ludwigia perennis* (IVI – 3.63)	*Nymphaea pubescens* (IVI- 10.81)	*Hydrilla verticillata* (IVI – 18.29)
W7	2.87 – 17.20	*Colocasia esculenta* (IVI - 2.87)	*Rotala rotundifolia* (IVI- 9.97)	*Pontederia crassipes* (IVI – 17.20)

biomass production, creating surface area for gas exchange and improving water quality. For fodder production, species such as *Alternanthera philoxeroides*, *Cyperus iria*, *Panicum repens*, and *Paspalum distichum*, exhibit high biomass production and dense vegetation cover, making them valuable as palatable fodder for livestock while contributing to the stability and productivity of wetland ecosystems.

Discussion

The study found a diverse array of macrophyte species across these seven wetlands (W1-W7), with emergent plants dominating (69%), aligning with Vymazal's (2013) findings on free-water surface-constructed wetlands. The presence of all four growth forms indicates a complex ecosystem with diverse microhabitats crucial for supporting aquatic fauna and ecosystem functions (Jeppesen et al., 2012). The low percentage of submerged species (11%) might suggest water clarity or quality issues, as noted by Blindow et al. (2000). Localized dominant macrophytes play vital roles in wetland ecosystems. Species like *Lemna perpusilla*, *Spirodela polyrhiza, Bacopa monnieri*, and *Azolla pinnata* thrive in specific environmental conditions. *L. perpusilla* and *S. polyrhiza* prefer shallow, nutrient-rich waters with high light intensity and warm temperatures (Smith and Barko, 1990; Brock, 1997). *B. monnieri* adapts to various light conditions in shallow depths (Smith, 2014), while *A. pinnata* tolerates diverse water depths and can thrive in nutrient-poor waters due to its nitrogen-fixing ability

Table 7. Traits and ecosystem services of key species.

Tabela 7. Lastnosti in ekosistemske storitve ključnih vrst.

Traits of the macrophytes	Name of the macrophytes
Soil erosion control, soil stabilization and nutrient absorption	
Extensive root system Root surface area Root hair density Root length, root depth Root exudates Mycorrhizal associations	*Alternanthera philoxeroides, Bacopa monnieri, Cyperus iria,* *Cyperus difformis, Cyperus compressus, Echinochloa colonum,* *Fimbristylis bisumbellata, Fimbristylis argentea,* *Panicum repens, Paspalum distichum, Rotala rotundifolia,* *Pistia stratiotes, Potamogeton crispus*
Sediment Retention	
Dense root network Fibrous roots fine root hairs Rapid root growth Root depth Rhizome formation Above ground biomass	*Alternanthera philoxeroides,* *Bacopa monnieri, Potamogeton crispus,* *Azolla pinnata, Lemna perpusilla, Pistia stratiotes,* *Nymphoides hydrophyllum, Trapa natans var. bispinosa,* *Pontederia crassipes, Spirodela polyrhiza,* *Hydrilla verticillate, Fimbristylis bisumbellata*
Water Quality Improvement	
Root exudates and high nutrient uptake Dense root network and pollutant absorption High biomass production and mycorrhizal associations Surface area for gas exchange	*Alternanthera philoxeroides, Hydrilla verticillate,* *Lemna perpusilla, Azolla pinnata, Spirodela polyrhiza,* *Nymphoides hydrophyllum, Pistia stratiotes,* *Pontederia crassipes, Potamogeton crispus,* *Trapa natans var. bispinosa, Bacopa monnieri,* *Rotala rotundifolia*
Fodder	
High biomass production Palatability for livestock High biomass Dense vegetation cover	*Alternanthera philoxeroides, Cyperus iria, Cyperus difformis,* *Cyperus compressus, Echinochloa colonum,* *Fimbristylis bisumbellata, Fimbristylis argentea,* *Panicum repens, Paspalum distichum*

(Lumpkin and Plucknett, 1982). These species significantly contribute to wetland biodiversity and ecological dynamics. The dominance of Cyperaceae (20.51%) is common in tropical and subtropical wetlands (Gopal, 2016). Sedge-dominated vegetation supports food webs, creates habitats, and removes toxic compounds from water. It provides food for insects, birds, and mammals, with some species utilized by humans (Mishra et al., 2016). Cyperaceae species are well-adapted to wetland conditions and play crucial roles in nutrient cycling and sediment stabilization. The significant presence of Poaceae (6.41%) further emphasizes the importance of graminoid species. The diversity of other dominant families (Araceae, Polygonaceae, Hydrocharitaceae), including existing families suggests a complex wetland flora supporting various ecological niches, potentially indicating overall health and resilience to environmental changes (Mitsch and Gosselink, 2015). Further research is needed to understand the specific ecological roles and interactions of these families within the studied wetlands.

The species frequency analysis reveals distinct distribution patterns across the wetlands. High-frequency species like *Pontederia crassipes, Alternanthera philoxeroides*, and *Hydrilla verticillata* demonstrate adaptability to various conditions, with A. philoxeroides being a known invasive species (https://www.iucngisd.org/gisd/). The abundance of *Nymphoides hydrophyllum* and *N. indica* aligns with Preston and Croft's (2022) findings on species with effective reproductive strategies. High frequencies of *Pistia stratiotes* and *Potamogeton crispus* may indicate nutrient enrichment (Guntenspergen et al., 2020), potentially due to wastewater inflow. Low-frequency species like *Acmella paniculata* and *Torenia crustacea* show localized distributions, suggesting specific habitat requirements or less competitiveness (Mishra et al., 2016). The restricted distribution of *Bacopa monnieri* and *Persicaria glabra* indicates potential habitat specificity or vulnerability to competition and disturbance. Urban wetlands face significant anthropogenic pressures affecting sensitive species (Grime, 2001). *Azolla pinnata, Lemna perpusilla,* and *Spirodela polyrhiza*, observed with low frequencies in W6, may be restricted due to environmental factors (Gopal, 2015). The species range reflects wetland ecosystem complexity, with high-frequency species shaping habitats and supporting ecosystem functions, while low-frequency species contribute to biodiversity and resilience (Mishra et al., 2016).

In comparison to Raunkaier's law of frequency classes (1934), all the wetlands (W1-W7) revealed heterogeneous vegetation structures, consistent with similar research conducted in the Hokersar wetland in the Kashmir Himalaya (Mir et al., 2009). W1 displayed an even species distribution, suggesting a more stable ecosystem likely resulting from balanced environmental conditions and minimal extreme disturbances. Similarly, sites W4 and W7 showed some degree of balance, contributing to ecological resilience. The importance of species distribution and evenness for ecosystem stability in wetlands is further emphasized by the work of Mälson et al. (2008). Their study on rich fen vegetation revealed that drainage significantly reduced biodiversity, while the slow recovery observed post-rewetting underscores the critical role of maintaining evenness for ecosystem resilience. Conversely, W2, W3, W5, and W6 exhibited complex patterns of species dominance and potential instability, with deviations from Raunkaier's normal frequency. Particularly, sites W2 and W5 exhibited notable species dominance, with W2 showing a higher frequency in class B than Raunkaier's normal frequency, while W5 displayed a decrease in class A, increases in classes B and C, and an absence of class E. These patterns suggest less stable ecosystems facing extreme conditions and competitive interactions (Tilman, 1982). Zedler and Kercher (2005) highlighted wetlands' vulnerability to invasions, noting that 24% of the world's most invasive plants are wetland species. They attributed this to wetlands acting as landscape sinks accumulating debris, sediments, water, and nutrients conditions that can facilitate invasions by creating canopy gaps or promoting opportunistic plant growth. Luo et al. (2010) demonstrated that intraspecific and interspecific competition influence biomass accumulation nutrient accumulation in marsh plants, with competition strongest at lower water levels, while facilitation is more pronounced at higher water levels. The analysis reveals the complex interplay of environmental factors, competitive interactions, and ecosystem stability in these wetlands, emphasizing the utility of Raunkaier's frequency classes in understanding wetland ecosystems and the importance of conservation efforts to maintain species evenness for long-term ecosystem stability.

The analysis of four different biodiversity indices across seven wetlands (W1-W7) provides a comprehensive understanding of ecosystem health, species richness, and distribution patterns. The Shannon-Wiener diversity index (H') revealed varying levels of species complexity, with W7 exhibiting the highest biodiversity (3.4968) and W2 the lowest (2.9512). This pattern aligns with studies on the Hanjiang River wetlands, which demonstrated varied H' values

based on plant community structure and human impact (Li et al., 2022). Simpson's index of diversity (1-D) corroborated these findings, showing high values (0.9505 to 0.9654) for W1, W3, W4, W6, and W7, indicating diverse and stable ecosystems. However, W2 and W5 displayed slightly lower (1-D) values (0.9367 and 0.9464, respectively), suggesting potential biodiversity reduction or species dominance, consistent with findings on the impact of urbanization in the Bamenda Municipality (Asongwe et al., 2022). The Margalef Diversity Index (DMg) provided insights into species richness, with W1, W3, W4, W6, and W7 showing relatively high values (3.887354 to 4.207085), indicating rich species diversity and favourable conditions for plant life. These high values likely reflect optimal environmental conditions and effective conservation efforts, aligning with the diverse and healthy ecosystems observed in Lake Alakol (Zaparina et al., 2024). Conversely, W2 (3.853248) and W5 (3.628268) exhibited lower DMg values, suggesting reduced species richness, possibly due to environmental stressors such as pollution, human activities, or invasive species (Saluja and Garg, 2017). These findings mirror challenges faced by ecosystems impacted by anthropogenic influences, similar to observations in the tropical lakes of the Central Ganga Plain (Dubey et al., 2022). Pielou's Evenness (J') analysis revealed high evenness values for W3, W4, W5, W6, and W7 (0.94020 to 0.96965), indicating stable environmental conditions with minimal species dominance. Interestingly, while W5 showed lower values in other indices, it maintained high evenness, suggesting a relatively balanced distribution of species despite potential reductions in overall diversity. W1 (0.92904) and W2 (0.91683) displayed lower evenness, aligning with their performance in other indices. These findings are consistent with Zhao et al. (2021), who reported that farmland abandonment positively influences evenness by transitioning plant communities from annual to perennial species. The lower values of W1 and W2, as noted by Sharma and Singh (2017), may reflect the effects of seasonal fluctuations and vegetation on evenness. The combined analysis of these four biodiversity indices paints a nuanced picture of the seven wetlands' ecological health. W7 consistently emerges as the most diverse and stable ecosystem, followed closely by W3, W4, and W6. These wetlands likely benefit from optimal environmental conditions and effective conservation efforts. W5 presents an interesting case, with lower diversity and richness but maintaining high evenness, suggesting a need for targeted conservation strategies to enhance its overall biodiversity while preserving its balanced species distribution. W1 and W2 consistently show signs of ecological stress across all indices, indicating they are prime candidates for intensive conservation and restoration efforts. These findings underscore the importance of comprehensive biodiversity assessments in wetland management and highlight the need for tailored conservation strategies that address the specific challenges faced by each wetland ecosystem. Long-term monitoring, such as that conducted in the Yellow River Delta since 2001, has shown that restored wetlands can significantly improve water quality and soil conditions, with vegetation quickly re-establishing and providing habitat for diverse bird species (Cui et al., 2009). Implementing such targeted conservation strategies could help improve diversity in degraded wetlands and ensure long-term ecological health across various wetland ecosystems.

The Important Value Index (IVI) of each macrophyte of the studied wetlands reveals significant patterns in species composition and ecological importance (Mori et al., 2015). The findings indicate a clear stratification of species based on their IVI values: Low IVI species, such as *Acmella paniculata* and *Hydrilla verticillata*, exhibit limited ecological roles but contributed to overall biodiversity (Junk et al., 2013). The majority of species, comprising 58-77% across study sites (W1-W7), demonstrated intermediate IVI values. These species, including *Ipomoea aquatica* and *Nymphoides indica*, play crucial roles in maintaining ecological stability and highlight the complexity of wetland ecosystems. Of particular concern are the high IVI species, approximately 21 in total, including *Alternanthera philoxeroides, Pontederia crassipes,* and *Pistia stratiotes*, which emerge as key invasive components of the wetland ecosystems (Zedler and Kercher, 2005). These dominant species, while indicative of successful adaptation to the wetland environment, raise concerns about their potential negative impacts on native biodiversity and ecosystem functions, necessitating careful monitoring and management strategies. The dominance of emergent species, followed by submersed, rooted floating-leaves, and free-floating growth forms, aligns with other wetland studies (Junk et al., 2013). This pattern suggests possible terrestrialization or eutrophication processes (Mitsch and Gosselink, 2015). While IVI provides valuable insights, it has limitations in capturing the functional roles of less abundant species (Frieswyk et al., 2007). The study emphasizes the need for balanced management strategies that consider both high and low IVI species to maintain biodiversity and ecosys-

tem resilience (Zedler and Kercher, 2005). This analysis provides a comprehensive understanding of wetland plant community structure and species importance, highlighting the need for accurate, adaptive management approaches. Future research should focus on long-term monitoring and integration of functional trait analyses with IVI data to gain deeper insights into ecosystem processes and community assembly mechanisms in these wetland ecosystems. The identification of nine rare species is significant for conservation. Species like *Grangea maderaspatana* and *Scleromitrion brachypodum*, with higher IVI values among rare species, are of particular interest. Their rarity may be due to specific habitat requirements or environmental sensitivity (Grime, 2001). Rare submerged species such as *Ottelia alismoides* and *Ceratopteris thalictroides* are noteworthy, as they are often sensitive to water quality and habitat changes (Carpenter and Lodge, 1986). Their low abundance could indicate ecosystem stress, possibly from urbanization and pollution in wetlands W2 and W5. Elo et al. (2018) examined how environmental factors and human activities affect aquatic macrophyte diversity and rarity. The rarity of *Pouzolzia zeylanica* in W2, potentially due to habitat degradation and pollution, emphasizes the need for targeted conservation strategies. Salgado et al. (2023) found that protected land enhances native aquatic macrophyte survival and limits invasive species' spread. Monitoring and managing the impact of invasive species like *Pontederia crassipes* on rare species populations is crucial for maintaining ecological balance.

The study has highlighted the crucial role of plant functional traits, particularly root traits, in delivering various ecosystem services (Faucon et al., 2017; Bardgett et al., 2014; Burylo et al., 2012). Root traits contribute significantly to various ecosystem functions. For instance, sediment retention is enhanced by dense root networks, fine root hairs, rhizome formation, rapid root growth, fibrous roots, and substantial aboveground biomass (Stokes et al., 2009; Borin et al., 2005; Blanco-Canqui et al., 2004). In terms of water quality improvement, macrophytes exhibit high nutrient uptake, pollutant absorption, root exudates, high biomass production, and mycorrhizal associations (Vymazal, 2007). Furthermore, erosion control and soil stabilization are facilitated by extensive root systems and high root hair density. Based on these findings, macrophytes such as *Bacopa monnieri, Alternanthera philoxeroides, Cyperus spp., Echinochloa colonum, Fimbristylis spp., Panicum repens, Paspalum distichum, Rotala rotundifolia, Pistia stratiotes*, and *Pontederia crassipes* exhibit traits such as extensive root systems and high root hair density. These traits enable these species to provide essential ecosystem services, including erosion control, soil stabilization, and nutrient absorption. In addition to these services, they also offer wildlife habitat, food, and fodder. Aquatic macrophytes play a crucial role in wastewater treatment and the management of algal blooms by providing essential ecosystem services. The provisioning services they offer include direct benefits such as nutrient removal and biomass production, which significantly improve water quality and create habitats for aquatic organisms. For example, *Azolla pinnata* excels in nutrient uptake and pollutant absorption (Rai, 2008), while *Lemna perpusilla* rapidly absorbs nutrients, effectively mitigating eutrophication (Vymazal, 2007). Additionally, regulating services are evident in how these species help to maintain ecological balance. *Hydrilla verticillata*, with its high root hair density that increases surface area, is particularly effective in treating nutrient-rich wastewater (Dhir, 2013), and P*ontederia crassipes* contributes high biomass production that aids in pollutant absorption (Raven et al., 2005). Furthermore, *Spirodela polyrhiza* supports nutrient cycling and overall ecosystem health through its rapid nutrient uptake (Cronk and Fennessy, 2016). Collectively, these species not only enhance water quality but also foster biodiversity within aquatic ecosystems. Similarly, *Potamogeton crispus, Hydrilla verticillata* also provides habitat and food resources for various aquatic organisms. In terms of provisioning services, species like *Azolla pinnata, Lemna perpusilla, Pistia stratiotes*, and *Spirodela polyrhiza* are utilized as animal feed and biofertilizers, promoting agricultural sustainability. Furthermore, *Nymphoides hydrophyllum, Marsilea minuta* and *Ipomoea aquatica* serve as fodder for livestock, with *M. minuta* and *Ipomoea aquatica* also being consumed as a vegetable. Traditional medicinal practices utilize plants such as *M. minuta* and *Bacopa monnieri* for their therapeutic properties. Additionally, *Trapa natans* var. *bispinosa* produces edible seeds that are valuable for both human diets and traditional medicine.

Conclusion

This pioneering study in the Eastern Ranchi District of Jharkhand, India, identified 78 macrophyte species across seven wetlands (W1-W7), revealing a predominance of

emergent species (69%) and underscoring the richness of these ecosystems. The analysis documented nine rare species and 21 key species that contribute significantly to essential ecosystem services like soil stabilization and water quality improvement. High-frequency species such as *Pontederia crassipes* (85.71% in W1) and *Hydrilla verticillata* (57.14% in W1) demonstrated resilience across diverse habitats, while low-frequency species like *Acmella paniculata* (14.29% in W1) were limited to specific areas due to habitat sensitivity. Raunkiaer's frequency analysis indicated a dominance of Class B species in most wetlands, particularly W1 (64.52%), while W2 exhibited signs of disturbance with a higher proportion of Class A species (52%) and fewer in Classes C and E. Wetland W7 had the highest biodiversity (H' = 3.4968) and richness (DMg = 5.08069), while W2 had the lowest values, and Wetland W6 showed the highest evenness (J' = 0.96965), indicating ecological stability. Intermediate IVI species dominated most wetlands (58–77%), with *Pontederia crassipes* (IVI = 20.72) prominent in W1, and *Acmella paniculata* (IVI = 3.12) less so. This study provides essential baseline data for developing targeted conservation strategies to address ecological stress in urban wetlands (W1, W2, and W5). It emphasizes the crucial role of macrophytes in sustaining ecosystem services, highlighting their importance for wetland conservation and biodiversity management.

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Author's contributions

Conceptualization, S.G.; methodology, J.M.; data curation, J.M.;formal analysis, J.M.; manuscript preparation—original draft preparation, J.M.; supervision, S.G.; visualization, S.G.; writing—review and editing, S.G.All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References

Asongwe, G. A., Bame, I. B., Ndam, L. M., Orock, A. E., Tellen, V. A., Bumtu, K. P., Tening, A. S., 2022. Influence of urbanization on phytodiversity and some soil properties in riverine wetlands of Bamenda municipality, Cameroon. Scientific Reports, 12(1), 19766. https://doi.org/10.1038/s41598-022-23278-7

Bardgett, R. D., Mommer, L., De Vries, F. T., 2014. Going underground: root traits as drivers of ecosystem processes. Trends in ecology & evolution, 29(12), 692-699. https://doi.org/10.1016/j.tree.2014.10.006

Bhanja, A., Sinha, N., Mandal, B., Payra, P., 2023. Diversity of Aquatic Macrophytes in Four Blocks of Purba Medinipur District, West Bengal, India. Ind. J. Pure App. Biosci, 11(1), 1-8. http://dx.doi.org/10.18782/2582-2845.8974

Blanco-Canqui, H., Gantzer, C. J., Anderson, S. H., Alberts, E. E., Thompson, A. L., 2004. Grass barrier and vegetative filter strip effectiveness in reducing runoff, sediment, nitrogen, and phosphorus loss. Soil Science Society of America Journal, 68(5), 1670-1678. https://doi.org/10.2136/sssaj2004.1670

Blindow, I., Hargeby, A., Wagner, B.M., Andersson, G., 2000. How important is the crustacean plankton for the maintenance of water clarity in shallow lakes with abundant submerged vegetation?. Freshwater Biology, 44(2), pp.185-197. https://doi.org/10.1046/j.1365-2427.2000.00552.x

Borin, M., Vianello, M., Morari, F., Zanin, G., 2005. Effectiveness of buffer strips in removing pollutants in runoff from a cultivated field in North-East Italy. Agriculture, ecosystems & environment, 105(1-2), 101-114. https://doi.org/10.1016/j.agee.2004.05.011

Brock, M. A., 1997. Plant life at the edges of wetlands: ecological responses to wetting and drying patterns. Frontiers in ecology: building the links, 181-192.

Burylo, M., Rey, F., Mathys, N., Dutoit, T., 2012. Plant root traits affecting the resistance of soils to concentrated flow erosion. Earth Surface Processes and Landforms, 37(14), 1463-1470. https://doi.org/10.1002/esp.3248.

Carpenter, S. R., Lodge, D. M., 1986. Effects of submersed macrophytes on ecosystem processes. Aquatic botany, 26, 341-370. https://doi.org/10.1016/0304-3770(86)90031-8

Chowdhury, M., Chowdhury, A., 2022. "Wetland Flora of West Bengal," Bluerose Publishers, Pvt. Ltd. Pp.1-354.

Cook, C. D. K., 1996. Aquatic and Wetland Plants of India. Oxford University Press, New York. https://doi.org/10.1093/oso/9780198548218.001.0001

Cronk, J. K., Fennessy, M. S., 2016. Wetland plants: biology and ecology.1st ed. CRC Press: Boca Raton, Florida, USA. https://doi.org/10.1201/9781420032925

Cui, B., Yang, Q., Yang, Z., Zhang, K., 2009. Evaluating the ecological performance of wetland restoration in the Yellow River Delta, China. Ecological Engineering, 35(7), 1090-1103. https://doi.org/10.1016/j.ecoleng.2009.03.022

Dhir, B., 2013. Phytoremediation: role of aquatic plants in environmental clean-up (Vol. 14, pp. 1-111). New Delhi: Springer. https://doi.org/10.1007/978

Dubey, D., Kumar, S., Dutta, V., 2022. Impact of nutrient enrichment on habitat heterogeneity and species richness of aquatic macrophytes: evidence from freshwater tropical lakes of Central Ganga Plain, India. International Journal of Environmental Science and Technology, 19(6), 5529-5546. https://doi.org/10.1007/s13762-021-03438-4

Elo, M., Alahuhta, J., Kanninen, A., Meissner, K.K., Seppälä, K., Mönkkönen, M., 2018. Environmental characteristics and anthropogenic impact jointly modify aquatic macrophyte species diversity. Frontiers in Plant Science, 9, p.1001.

Faucon, M. P., Houben, D., Lambers, H., 2017. Plant functional traits: soil and ecosystem services. Trends in plant science, 22(5), 385-394. https://doi.org/10.1016/j.tplants.2017.01.005

Frieswyk, C.B., Johnston, C.A., Zedler, J.B., 2007. Identifying and characterizing dominant plants as an indicator of community condition. Journal of Great Lakes Research, 33, 125-135. https://doi.org/10.3394/0380-1330(2007)33[125:IACDPA]2.0.CO;2

Gopal, B., 2016. Should 'wetlands' cover all aquatic ecosystems and do macrophytes make a difference to their ecosystem services?. Folia Geobotanica, 51(3), 209-226. https://doi.org/10.1007/s12224-016-9248-x

Grime, J. P., 2001. Plant strategies, vegetation processes, and ecosystem properties. 2nd ed. John Wiley & Sons Inc., (NY). p 417.

Guntenspergen, G. R., Stearns, F., Kadlec, J. A., 2020. Constructed wetlands for wastewater treatment, 1st ed., Donald A. Hammer, CRC Press: Boca Raton Florida, USA. https://doi.org/10.1201/9781003069850

Jeppesen, E., Søndergaard, M., Lauridsen, T.L., Davidson, T.A., Liu, Z., Mazzeo, N., Trochine, C., Özkan, K., Jensen, H.S., Trolle, D., Starling, F., 2012. Biomanipulation as a restoration tool to combat eutrophication: recent advances and future challenges. Advances in ecological research, 47, 411-488. https://doi.org/10.1016/B978-0-12-398315-2.00006-5

Junk, W.J., An, S., Finlayson, C.M., Gopal, B., Květ, J., Mitchell, S.A., Mitsch, W.J., Robarts, R.D., 2013. Current state of knowledge regarding the world's wetlands and their future under global climate change: a synthesis. Aquatic sciences, 75, 151-167. https://doi.org/10.1007/s00027-012-0278-z

Kumar, S., Singh, R., Kumar, D., Bauddh, K., Kumar, N., Kumar, R., 2023. An Introduction to the Functions and Ecosystem Services Associated with Aquatic Macrophytes. In Aquatic Macrophytes: Ecology, Functions and Services (pp. 1-20). Singapore: Springer Nature Singapore. https://doi.org/10.1007/978-981-99-3822-3_1

Lal, A., Lal, N., 2021, Studies on Macrophytic diversity of two major water bodies of Ranchi, Viz. Ranchi lake and Kanke Dam, Ranchi, Jharkhand The Biobrio, 8(3 & 4), 711-715.

Lesiv, M. S., Polishchuk, A. I., Antonyak, H. L., 2020. Aquatic macrophytes: ecological features and functions. Studia Biologica, 14(2), 79-94. http://dx.doi.org/10.30970/sbi.1402.619

Li, Z., Zhang, Y., Li, F., Duan, M., 2022. Plant Diversity and Quantitative Characteristics Of Wetland Community on the North Bank of Hanjiang River in Cheng GU, Shaanxi Province, China. Bangladesh Journal of Botany, 839-848. https://doi.org/10.3329/bjb.v51i40.63825

Lumpkin, T. A., Plucknett, D. L., 1982. Azolla as a green manure: Use and Management in crop production, Press, Hawaii Univ., Honolulu, Hawaii, HI 96822, USA pp. 230.

Luo, W., Xie, Y., Chen, X., Li, F., Qin, X., 2010. Competition and facilitation in three marsh plants in response to a water-level gradient. Wetlands, 30(3),525-530. https://doi.org/10.1007/s13157-010-0064-4

Magurran, A.E., 2004. Measuring biological diversity. Blackwell Science, Oxford, UK.

Mälson, K., Backéus, I., Rydin, H., 2008. Long-term effects of drainage and initial effects of hydrological restoration on rich fen vegetation. Applied Vegetation Science, 11(1), 99-106. https://doi.org/10.1111/j.1654-109X.2008.tb00208.x

Margalef, R., 1969. Diversity and stability: a practical proposal and a model of interdependence. Brookhaven Symposia in Biology. 22, 25-37. http://hdl.handle.net/10261/166352

Mir, A. A., Mahajan, D. M., Saptarishi, P. G., 2009. Composition and distribution of macrophytes in Hokersar-A wetland of international importance in Kashmir Himalaya. International Journal of Climate Change: Impacts and Responses, 1(4), 23-35.

Mishra, A., Lal, B., 2023. Assessment of groundwater quality in Ranchi district, Jharkhand, India, using water evaluation indices and multivariate statistics. Environmental Monitoring and Assessment, 195(4), 472.

Mishra, S., Tripathi, A., Tripathi, D.K., Chauhan, D.K., 2016. Role of sedges (Cyperaceae) in wetlands, environmental cleaning and as food material: Possibilities and future perspectives. Plant-environment interaction: Responses and approaches to mitigate stress, pp.327-338. https://doi.org/10.1002/9781119081005.ch18

Mitsch, W. J., Bernal, B., Hernandez, M. E., 2015. Ecosystem services of wetlands. International Journal of Biodiversity Science, Ecosystem Services & Management, 11(1), 1-4..https://doi.org/10.1080/21513732.2015.1006250

Mori, A.S., Fujii, S., Kitagawa, R., Koide, D., 2015. Null model approaches to evaluating the relative role of different assembly processes in shaping ecological communities. Oecologia, 178, 261-273. https://doi.org/10.1007/s00442-014-3170-9

Mukherjee, P., Kumar, J., 2020. Floristic Studies on Aquatic and Semi-aquatic Angiosperms of Major Water Bodies of Jharkhand. Journal of Indian Botanical Society, 100(3&4): 119-133. http://dx.doi.org/10.5958/2455-7218.2020.00032.7

POWO., 2024: Plants of the World Online Facilitated by the Royal Botanic Gardens, Kew. Published on the internet; http://www.plantsoftheworldonline.org. Retrieved 30 August 2024.

Preston, C. D., Croft, J. M., 2022. Aquatic plants in Britain and Ireland. Brill, Harley Books, Coichester, Essex, England.

Rai, P. K., 2008. Phytoremediation of Hg and Cd from industrial effluents using an aquatic free floating macrophyte Azolla pinnata. International journal of phytoremediation, 10(5), 430-439. https://doi.org/10.1080/15226510802100606

Rani, M., Kumar, P., Yadav, M., Hooda, R. S., 2011. Wetland Assessment and Monitoring Using Image Processing Techniques: A Case Study of Ranchi, India. Journal of Geographic Information System. 3. 345-350. https://doi:10.4236/jgis.2011.34032

Raunkiaer, C., 1934. The life forms of plants and statistical plant geography; being the collected papers of C. Raunkiær, Oxford: Clarendon Press., p 632.

Raven, P. H., Evert, R. F., 2005. Eichhorn SE. Biology of Plants. W. H. Freeman and Company Publishers, New York, pp 900. http://dx.doi.org/10.1093/aob/mcu090.

Salgado, J., Vélez, M.I., González-Arango, C., O'Dea, A., 2023. Protected land enhances the survival of native aquatic macrophytes and limits invasive species spread in the Panama Canal. Aquatic Conservation: Marine and Freshwater Ecosystems, 33(7), pp.737-750. https://doi.org/10.1002/aqc.3970

Saluja, R., Garg, J. K., 2017. Macrophyte species composition and structure along littoral region in relation to limnological variables of a tropical wetland ecosystem. Chemistry and Ecology, 33(6), 499-515. https://doi.org/10.1080/02757540.2017.1328502

Sharma, R. C., Singh, S., 2017. Macrophytes of sacred Himalayan Lake Dodi Tal, India: quantitative and diversity analysis. Biodiversity International Journal, 1(4), 1-11. https://DOI: 10.15406/bij.2017.01.00020

Shiji, M., Sabitha, A. R., Prabhakar, K., Harikumar, P. S., 2016. Water quality assessment of Kavvayi Lake of northern Kerala, India using CCME water quality index and biological water quality criteria. Journal of Environmental biology, 37(6), 1265-72.

Smith, C. S., Barko, J. W., 1990. Ecology of Eurasian watermilfoil. Journal of Aquatic Plant Management, 28(2), 55-64.

Smith, S. D., 2014. The roles of nitrogen and phosphorus in regulating the dominance of floating and submerged aquatic plants in a field mesocosm experiment. Aquatic Botany, 112, 1-9. https://doi.org/10.1016/j.aquabot.2013.07.001

Stokes, A., Atger, C., Bengough, A. G., Fourcaud, T., Sidle, R. C., 2009. Desirable plant root traits for protecting natural and engineered slopes against landslides. Plant and soil, 324, 1-30. https://doi.org/10.1007/s11104-009-0159-y

Swamy, J., Chandramohan, K., Bhadraiah, B., 2016. An inventory of aquatic and wetland plants of Pocharam Lake, Medak district, Telangana, India. Asian Journal of Plant Science and Research, 6(3), 87-91.

Thomaz, S. M., 2023. Ecosystem services provided by freshwater macrophytes. Hydrobiologia, 850(12), 2757-2777. https://doi.org/10.1007/s10750-021-04739-y

Tilman, D., 1982. Resource competition and community structure (No. 17). Princeton university press. p 296.

Verma, S. K., Pandeya, P. K. 2008. Floristic studies of Aquatic and Semi aquatic Angiosperms of Ratu Maharaja Pond, Ranchi, Jharkhand. Biospectra. 2(1): 139-41. www.theplantlist.org.

Vymazal, J., 2007. Removal of nutrients in various types of constructed wetlands. Science of the total environment, 380(1-3), 48-65. https://doi.org/10.1016/j.scitotenv.2006.09.014

Vymazal, J., 2013. Emergent plants used in free water surface constructed wetlands: a review. Ecological engineering, 61, pp.582-592. https://doi.org/10.1016/j.ecoleng.2013.06.023

WFO., 2024: World Flora Online. Published on the internet; http://www.worldfloraonline.org. Retrieved 30 August, 2024

Xu, T., Weng, B., Yan, D., Wang, K., Li, X., Bi, W.,....., Liu, Y., 2019. Wetlands of international importance: Status, threats, and future protection. International journal of environmental research and public health, 16(10), 1818. https://doi.org/10.3390/ijerph16101818

Zaparina, Y., Inelova, Z., Mengtay, A., Mukhitdinov, A., Boros, E., 2024. Ecological analysis of higher aquatic and semi-aquatic plants of Lake Alakol. In BIO Web of Conferences (Vol. 100, p. 04015). EDP Sciences. 100: 04015 (Article number). https://doi.org/10.1051/bioconf/202410004015

Zedler, J.B., Kercher, S., 2005. Wetland resources: status, trends, ecosystem services, and restorability. Annu. Rev. Environ. Resour., 30(1), 39-74. https://doi.org/10.1146/annurev.energy.30.050504.144248

Zhao, R., Zhang, X., Zhang, L., Wang, Y., 2021. Plant diversity and soil properties at different wetland restoration stages along a major river in the arid Northwest of China. Wetlands, 41(1), 13-22. https://doi.org/10.1007/s13157-021-01412-x

ABS

Original Research

The effectiveness of teaching human evolution to 14- to 15-year-olds in Slovenia

Jelka Strgar [1*]

Abstract

In Slovenia, human evolution is first taught in biology classes to 14–15-year-old students. For many, this will be the only contact with this topic at school. The objective was to determine how much knowledge students acquired about human evolution. Data were collected with a questionnaire using a 5-point Likert scale. The participants were 13–14-year-olds who had not yet learned about human evolution (the control group) and 15–16-year-olds who had learned about human evolution one year earlier in biology classes (the experimental group). The results show that students significantly improved their knowledge of human evolution with small to medium effect sizes. Students knew very well that modern humans are the only species of humans today and that they did not live at the same time as dinosaurs. The achievements were low on the following topics: modern humans lived at the same time as Neanderthals and mammoths, and modern humans did not evolve from Neanderthals. To the problematic topics, attention should be paid to teacher education and the biology curriculum. Few correlations were found between the knowledge of human evolution, acceptance of evolution, religiosity, and attitudes toward biology.

Keywords

knowledge of human evolution; acceptance of evolution; religiosity; attitudes toward biology; primary school

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Učinkovitost poučevanja evolucije človeka pri 14-do 15-letnikih v Sloveniji

Izvleček

V Sloveniji se pri biologiji evolucijo človeka prvič učijo 14- do 15-letniki. Za mnoge od njih bo to v šoli edini stik s to temo. Cilj raziskave je bil ugotoviti, koliko znanja evolucije človeka so učenci usvojili. Podatke smo zbrali z vprašalnikom s petstopenjsko Likertovo lestvico. Udeleženci so bili 13- do 14-letniki, ki se še niso učili evolucije človeka (kontrolna skupina) in 15- do 16-letniki, ki so se evolucijo človeka učili pri biologiji eno leto pred tem (poskusna skupina). Resultati kažejo, da so učenci signifikantno izboljšali svoje znanje evolucije človeka, in sicer z majhno do srednjo velikostjo učinka. Učenci so dobro vedeli, da je moderni človek danes edina vrsta človeka in da ni živel sočasno z dinozavri. Dosežki pa so bili nizki pri naslednjih temah: moderni človek je živel sočasno z neandertalcem in mamutom, moderni človek se ni razvil iz neandertalca. Problematičnim temam je treba posvetiti pozornost v izobraževanju učiteljev in osnovnošolskem učnem načrtu. Med znanjem evolucije človeka, sprejemanjem evolucije, vernostjo in odnosom do biologije je bilo malo korelacij.

Ključne besede

znanje evolucije človeka; sprejemanje evolucije; vernost; odnos do biologije; osnovna šola

Introduction

Human evolution is a topic that students can quickly lose interest in, especially if the teacher distracts them with too much theory and data that make no sense to them (Flammer, 2006). Motivation to learn about evolution is influenced by many factors, one of which is prior knowledge. Bloom and Weisberg (2007) claim that students' prior knowledge is the main reason for not accepting evolution, as misconceptions and beliefs can hinder the process of accepting new knowledge (Wescott & Cunningham, 2005; Smith, 2010; Yates & Marek, 2014). If the prior knowledge is good, the student can add new knowledge to the already existing one. However, if the student has misconceptions, motivation decreases, as it is extremely difficult to change entrenched misconceptions with classical teaching methods.

Slovenia ranks above average in the PISA survey. In the latest survey, which was conducted in 2022, Slovenian 15-year-olds ranked above average in scientific literacy. From these results, we can conclude that science literacy among primary and secondary school students in Slovenia is good, and it is also stable according to prior PISA surveys (Strgar, 2010; Štraus et al., 2016).

In a survey about the acceptance of the theory of evolution (Miller et al., 2006), Slovenia ranked in 16th place among 34 countries. University students in Slovenia think that understanding evolutionary concepts is a necessary part of solid general education, but there is also evidence that their actual understanding of evolution is not satisfactory. Surprisingly, they also think that alternative nonscientific theories of evolution should be presented in schools (Šorgo et al., 2014).

According to Pinxten et al. (2020), most of the population in many countries has a very limited formal introduction to the understanding of evolutionary theory. However, in most countries, there are national regulations that demand scientific teaching of evolutionary theory (Borgerding et al., 2016; Tavares & Bobrowski, 2018). In Slovenia, the theory of evolution was first introduced in school in 1945 (Vesel, 1977). According to the current national curriculum (Učni načrt. Program osnovna šola. Biologija, 2011), 14- to 15-year-old students learn about biological evolution in biology classes for the first time. However, some evolutionary concepts are already included in the curriculum for history that is taught to younger students (Učni načrt. Program osnovna šola. Zgodovina, 2011).

At the age of 14 to 15, students in Slovenia finish their compulsory primary education. Biological evolution is introduced to them in the final year (Učni načrt. Program osnovna šola. Biologija, 2011). There is one single national curriculum for all students up to this age. Afterwards, the students attend different secondary schools according to their career choices. These schools vary in the amount of biology courses that they offer. This means that some

of the students continue their education at secondary schools, which have no evolution as part of their curriculum. Into their adult life, these students take with them only the knowledge of evolution they had learned in primary school. Therefore, it is important to know what level of understanding of evolution the Slovenian school system provides during obligatory primary education.

In the Slovenian national curriculum for 14 to 15-year-olds, there are 18 objectives concerning evolution and only one objective regarding human evolution, namely, "Students can explain the origin of humans and primates and the kinship of humans with other primates" (Učni načrt. Program osnovna šola. Biologija, 2011, p. 17).

Published research shows that understanding evolution is challenging for students at all levels of education for a variety of reasons. Surroundings and socio-economic factors also influence students and take part in structuring students' preconceptions about evolution and their attitudes toward evolution (da Silva Oliveira et al., 2022). The problem, therefore, is how much students can learn at that age, specifically, how well they can understand evolutionary concepts and what contextual factors shape students' knowledge. Many studies (Miller et al., 2006; Hermann, 2013; Mantelas & Mavrikaki, 2020; da Silva et al., 2022; Salazar-Enriquez et al., 2023) confirmed a negative correlation between acceptance of evolution and religiosity. It differed depending on the country and cultural background of the people tested. For Slovenia, this problem was addressed in studies by Šorgo et al. (2014), Kralj et al. (2018), and Torkar and Šorgo (2020).

This research aimed to establish how much knowledge about human evolution students had before they learned about this topic in formal education and how much new knowledge about human evolution they acquired during formal education. Therefore, the aim was to determine how effective were the lessons on human evolution, which, according to the national curriculum for biology (Učni načrt. Program osnovna šola. Biologija, 2011), were attended by students aged 14 to 15 years. The point of interest was also whether contextual factors, such as attitudes toward biology, acceptance of evolution, and religiosity, were connected with students' knowledge of human evolution. The outcome could help make changes in the current national curriculum as well as be guidance for teacher educators and teachers themselves (Salazar-Enriquez et al., 2023). The following research questions were formulated:

RQ1: How effective are lessons about human evolution in Slovenia taught to 14–15-year-old students?

RQ2: Are the chosen contextual factors (students' acceptance of evolution, attitudes toward biology, and religiosity) connected with students' achievements on the knowledge test about human evolution?

Materials and Methods

General Background

The data were gathered in the 2021/22 and 2022/23 school years. The survey was anonymous; all the parents of the students signed an agreement to allow their children to participate. No benefits were offered to the participants. According to Slovenian legislation, such a survey does not need the approval of an ethics committee or similar body.

Sample

Our sample consisted of 593 students. The national curriculum for biology in Slovenia (Učni načrt. Program osnovna šola. Biologija, 2011) mandates for evolution to be taught to 14- to 15-year-olds. Students of this age were not part of the sample. In the survey, there were one year younger students (13- to 14-year-olds; a control group; n = 364).) who had not yet learned about human evolution. The experimental group (15- to 16-year-olds; n = 229) was represented by the students who had learned about human evolution in biology classes in the previous school year. In the sample, there was a slightly higher percentage (53.5%) of females compared to males.

Instrument and Procedures

Data were collected using a questionnaire. In the beginning, there were two demographic questions (gender and grade). These were followed by 59 statements that assessed students' knowledge of human evolution, their acceptance of evolution, attitudes toward biology, and religiosity. This part of the questionnaire used a 5-point Likert scale, ranging from strongly disagree (1) to strongly agree (5). Eleven items were originally worded in such a way that disagreement was required as a correct answer; these items were re-coded for statistical analysis. The questionnaire was compiled from statements used in several prior studies. The religiosity of participants was tested

with ten statements using the PERF scale, which was created by Beniermann (2019) and translated into the Slovenian language and used by Torkar and Šorgo (2020) on the Slovenian population. Eight statements that explored students' attitudes toward biology were adapted from The Relevance of Science Education study (Sjøberg & Schreiner, 2019). Seventeen statements that tested students' acceptance of evolution were taken from the MATE questionnaire (Rutledge & Sadler, 2007), and ten were adapted from the questionnaire used by Miller et al. (2006). The remaining 14 statements that tested students' knowledge about human evolution were partly adapted from the study by Miller et al. (2006) and partly designed in line with the curriculum and the textbooks that the participants of this study used when learning about evolution. Face validity of these 14 statements was ensured by the author and the three biology teachers who judged and revised them.

The questionnaire was administered during students' regular biology classes. Students completed it in approximately 15 minutes.

Data analysis

Data were tested for normal distribution with the Kolmogorov-Smirnov test; because the distribution of data was not normal ($ps < 0.001$), non-parametric testing was used. The statistical significance of differences in knowledge about human evolution between the control and the experimental groups was established by a Mann-Whitney U test. Effect size r was calculated using the equation $r = Z/\sqrt{N}$.

On 14 statements of the knowledge test about human evolution, descriptive statistics (mean, standard deviation) were used. On 45 statements assessing acceptance of evolution, attitudes toward biology, and religiosity, principal component analysis (PCA) was conducted using the orthogonal rotation (varimax) method with Kaiser normalization (Table S1). The value of the Kaiser-Meyer-Olkin measure of sampling adequacy was .925, which means that the sample size was adequate for PCA. Bartlett's test of sphericity was highly significant ($\chi2 = 11253.468$, $df = 990$, $p < .001$), indicating that correlations between statements were sufficiently large for PCA. According to the scree plot, retaining five components was justified. Following this, three statements were removed from the analysis. Thirteen statements loaded onto component 1 (religiosity); 14 statements loaded onto component 2 (acceptance of evolution – the theory); eight statements loaded onto component 3 (attitudes toward biology); four statements loaded onto component 4 (acceptance of evolution – the theory is (not) scientific), and three statements loaded onto component 5 (acceptance of evolution – organisms do (not) evolve). Cronbach's alphas for three components were highly reliable (from .85 to .91), while for two components, they were relatively less reliable (.53 and .68). These five components explained 55.926% of the variance. Cronbach's alpha for all 42 statements was .83. Additional results are available as supplementary material accompanying the online article.

Correlations between the knowledge of human evolution, acceptance of evolution, religiosity, and attitudes toward biology were analyzed using Spearman's correlation coefficient (rs). Comparisons of these correlations were then calculated to establish if the differences between correlations of the two students' age groups were statistically significant. For this, the following equation was used: $Zdifference = (zr1-zr2)/\sqrt{[1/(N1-3)]+[1/(N2+3)]}$ (Field, 2009, p. 191).

Results

Students' Achievements on the Knowledge Test about Human Evolution

Students' knowledge of human evolution was tested with 14 statements. Results show that all students showed the least knowledge ($M = 2.41$-2.57) in statements rE1 (Modern humans did not evolve from Neanderthals.), E2 (Modern humans lived in today's Slovenia at the same time as mammoths), and E36 (Modern humans and Neanderthals lived on Earth at the same time) (Tab. 1). In nine statements, they showed an average knowledge ($M = 3.03$-3.43). The highest knowledge ($M = 3.63$-4.13) was achieved on the following two statements: E3 (Today, all human species are extinct, except for our species, modern humans), rE38 (Modern humans did not live in today's Slovenia at the same time as dinosaurs.).

Differences among Students' Achievements on the Knowledge Test about Human Evolution by Age

On 12 out of 14 statements about the knowledge of human evolution, 13-14-year-olds answered significantly differently than 15-16-year-olds (Tab. 1). On one of those 12

statements (rE33 Modern humans did not evolve less than 15,000 years ago.), 13–14-year-olds showed more knowledge. On the remaining 11 statements, 15–16-year-olds had more knowledge. The effect sizes of differences in knowledge between 13–14-year-olds and 15–16-year-olds were mostly small (on 8 out of 14 statements) to medium (6 statements).

Correlations between Students' Achievements on the Knowledge Test about Human Evolution and Contextual Factors

Correlations for the subsample of 13–14-year-olds and the subsample of 15–16-year-olds were established, respectively. The variables were the average achievement on

Table 1. Differences in the students' knowledge of human evolution and effect sizes of differences.

Tabela 1. Razlike v znanju učencev o evoluciji človeka in velikost učinka razlik.

	Age (years)									
	All students (*N* = 589)		13-14 (*n* = 360)		15-16 (*n* = 229)		Mann-Whitney U test			Effect Size
Statement	**M**	**SD**	**M**	**SD**	**M**	**SD**	**U**	**Z**	***p***	**r**
rE1 Modern humans did not evolve from Neanderthals.*	2.41	1.229	2.29	1.183	2.60	1.276	35473.500	-2.742	.006	-.11
E2 Modern humans lived in today's Slovenia at the same time as mammoths.	2.51	1.237	2.44	1.248	2.62	1.215	36871.000	1.780	.075	.07
E36 Modern humans and Neanderthals lived on Earth at the same time.	2.57	1.348	2.43	1.267	2.79	1.441	33830.000	-2.933	.003	-.12
rE33 Modern humans did not evolve less than 15,000 years ago.*	2.99	1.045	3.06	1.060	2.89	1.015	36477.000	-2.108	.035	-.09
E34 Modern humans evolved in Africa.	3.03	1.086	2.78	1.084	3.43	.964	27174.500	-7.220	<.001	-.30
rE35 Humans did not evolve from chimpanzees.	3.08	1.183	2.81	1.102	3.49	1.185	28028.000	-6.466	<.001	-.27
E4 Neanderthals and modern humans share 99.7% of their genes.	3.14	.967	3.03	.924	3.32	1.008	33892.500	-3.532	<.001	-.15
E19 Neanderthals lived in Europe, Asia, and the Middle East.	3.30	.921	3.22	.962	3.41	.843	35868.500	-2.478	.013	-.10
E37 Humans's three closest relatives in terms of evolutionary development are gorillas, orangutans, and chimpanzees.	3.38	1.073	3.36	1.092	3.42	1.043	38414.500	-.774	.439	-.03
rE26 Humans today do not have more or less the same shape as we have always had.*	3.40	1.052	3.21	1.016	3.70	1.040	30096.500	-5.518	<.001	-.23
E41 Humans and chimpanzees evolved from a common ancestor.	3.42	1.089	3.22	1.060	3.74	1.062	29122.000	-6.138	<.001	-.25
rE5 The common ancestor of humans and chimpanzees does not live in Africa anymore.*	3.43	1.107	3.34	1.093	3.57	1.116	35846.000	-2.333	.020	-.10
E3 Today, all human species are extinct, except for our species, modern humans.	3.63	1.198	3.41	1.179	3.99	1.141	28594.500	-6.177	<.001	-.26
rE38 Modern humans did not live in today's Slovenia at the same time as dinosaurs.*	4.13	1.174	3.86	1.282	4.54	.823	28347.500	-6.678	<.001	-.28

*reversed statement

the knowledge test about human evolution and five components extracted by PCA: religiosity (F1), attitudes toward biology (F3), acceptance of evolution – the theory (F2), acceptance of evolution – the theory is (not) scientific (F4), and acceptance of evolution - organisms do (not) evolve (F5). There were six significant correlations altogether (Tab. 2, Tab. 3). Acceptance of evolution – the theory is (not) scientific (F4) was significantly correlated with religiosity (F1) in both subsamples and also with acceptance of evolution – the theory (F2) in 15–16-year-olds. Acceptance of evolution - organisms do (not) evolve (F5) was significantly correlated with attitudes toward biology (F3) in 15–16-year-olds. Knowledge of human evolution was significantly correlated with acceptance of evolution – the theory (F2) and attitudes toward biology (F3) in the group of 13–14-year-olds. All correlations were small (.13 < rs < .21).

Comparison of Correlations between the Control Group and the Experimental Group

A comparison of correlations between the control and the experimental group of students was calculated. The correlations between the knowledge of human evolution and the five components obtained by PCA in the group that had not yet learned about evolution (13–14-year-olds) were mostly significantly different (ps < .01) from the correlations in the group that had already learned about human evolution (15–16-year-olds). Only the correlation between acceptance of evolution (i.e., the theory is (not) scientific (F4) and acceptance of evolution - organisms do (not) evolve (F5)) was similar in both age groups (p = .223).

Discussion

The first research question dealt with whether teaching about human evolution to 14–15-year-old students in Slovenia is effective and whether it attains the standards set in the national curriculum for biology.

Overall, the average achievements on the knowledge test of human evolution ranged from very poor (M = 2.41) to very good (M = 4.31). In summary, Slovenian students knew very well that modern humans did not live at the same time as dinosaurs (rE38) and that modern humans are the

Table 2. Correlations between Students' Achievements on the Knowledge Test about Human Evolution and Contextual Factors (13–14-year-olds).
Tabela 2. Korelacije med dosežki učencev na testu znanja o evoluciji človeka in ozadenjskimi dejavniki (13-do 14-letniki).

Name of the Wetlands, (Blocks).	F1	F2	F3	F4	F5
F1 Religiosity	1.00				
F2 Acceptance of evolution – the theory	.06	1.00			
F3 Attitudes toward biology	.07	.00	1.00		
F4 Acceptance of evolution - the theory is (not) scientific	.16*	-.07	-.00	1.00	
F5 Acceptance of evolution - organisms do (not) evolve	.12	-.05	-.13	-.05	1.00
Knowledge of human evolution	-.08	.13*	-.18**	-.00	.03

* p < .05, ** p < .01

Table 3. Correlations between Students' Achievements on the Knowledge Test about Human Evolution and Contextual Factors (15–16-year-olds).
Tabela 3. Korelacije med dosežki učencev na testu znanja o evoluciji človeka in ozadenjskimi dejavniki (15-do 16-letniki).

Name of the Wetlands, (Blocks).	F1	F2	F3	F4	F5
F1 Religiosity	1.00				
F2 Acceptance of evolution – the theory	.04	1.00			
F3 Attitudes toward biology	-.05	-.07	1.00		
F4 Acceptance of evolution - the theory is (not) scientific	-.15*	.21**	.07	1.00	
F5 Acceptance of evolution - organisms do (not) evolve	.04	-.04	.20**	-.07	1.00
Knowledge of human evolution	-.07	-.04	.09	-.02	.02

* p < .05, ** p < .01

only species of humans today (E3). They also knew above averagely well that humans had changed their shape over time (rE26), that humans and chimpanzees evolved from a common ancestor (E41) that no longer lives (rE5), and that gorillas, orangutans and chimpanzees are humans' closest relatives (E37).

Students knew averagely well that modern humans evolved in Africa (E34), that they evolved more than 15,000 years ago (rE33), and that humans did not evolve from chimpanzees (rE35). In two of these statements, the knowledge improved significantly after the biology lessons in the ninth grade.

Students knew beyond average that modern humans and Neanderthals lived at the same time (E36), that modern humans lived at the same time as mammoths (E2), and that modern humans did not evolve from Neanderthals (rE1). For two of these statements, the biology lesson slightly improved knowledge.

In contrast, students were unsure what proportion of genes Neanderthals and modern humans share (E4) and where Neanderthals lived (E19), although knowledge improved significantly after the biology lessons in the ninth grade of primary school.

The fact that even the younger students had above-average achievements in seven out of 14 statements suggested that they might have gained some of this knowledge in history classes one year earlier (Učni načrt. Program osnovna šola. Zgodovina, 2011), through popular media (Modell & Wenderoth, 2005), digital and print media (Garibi et al., 2021), or their social surroundings (OECD, 2016; OECD, 2017; Šterman Ivančič & Mlekuž, 2023), especially their parents (Salazar-Enriquez et al., 2023).

The comparison of the knowledge of both groups showed that there were significant differences between students' answers in most of the statements. The experimental group (15–16-year-olds) showed mostly a better knowledge of human evolution than the control group (13–14-year-olds). Interestingly, younger students showed significantly better knowledge of one statement (rE33, Modern humans did not evolve less than 15,000 years ago). No knowledge was gained on the following two statements (E37) Human three closest relatives in terms of evolutionary development are gorillas, orangutans, and chimpanzees, and (E2) modern humans lived in today's Slovenia at the same time as mammoths. This suggested that these three topics were probably not addressed in biology classes. On the remaining 11 statements, older students answered significantly better. Therefore, we can conclude that in Slovenia, the teaching of (at least human) evolution at the primary school level is reasonably good. This is in line with Mead et al. (2017), who found that after classes, students' knowledge of evolution was improved immediately after the lesson and was retained. Some studies in other European countries showed that teaching evolution was not satisfactory and that evolution was taught only after the other topics had been covered (Pinxten et al., 2020). Some teachers also do not have sufficient content knowledge (Borgerding et al., 2015; Hartelt et al., 2022) and pedagogical skills to teach such a demanding topic (Stasinakis & Athanasiou, 2016). However, the fact that the effect sizes of all eleven improvements in knowledge of human evolution in the present study were mostly small or medium suggested that there is room for improvement in the way human evolution is taught. Changes in the curriculum should be made concerning the topics with low achievements. Despite some research not showing any connection between students' knowledge and the quality of the national curriculum (Belin & Kisida, 2015), other studies showed that the structure of the curriculum influences knowledge and acceptance of evolution (Vaughn & Robbins, 2017).

Information gathered in the present study can be applied immediately to improve the curriculum (Salazar-Enriquez et al., 2023), which is currently underway in Slovenia. The results will also be important for pre-service teachers' educational programs because by securing the understanding and acceptance of evolution in teachers, the transfer of these to new generations of students will be more favourable (Balgopal, 2014; Cofre et al., 2017; Torkar & Šorgo, 2020).

The second research question dealt with the chosen contextual factors (students' religiosity, attitudes toward biology, and acceptance of evolution) and their possible correlations with students' achievements on the knowledge test about human evolution. Six significant but small correlations were found.

According to most studies, the knowledge and acceptance of evolution are positively correlated (Carter et al., 2015; Tavares & Bobrowski, 2018; Torkar & Šorgo, 2020; Salazar-Enriquez et al., 2023). Surprisingly, there was no such correlation for 15–16-year-olds in the present study. However, for 13–14-year-olds, the results showed that the better knowledge they possessed, the more they accepted the general statements of the evolutionary theory (F2). This is in line with other studies indicating that the correlation

between knowledge and acceptance of evolution is not strong (Mead et al., 2017).

Most of the research (e.g., Salazar-Enriquez et al., 2023) found that knowledge and acceptance of evolution negatively correlate with religious beliefs. Interestingly, the present study showed no correlation between religiosity and knowledge. However, in both age groups, there was a small negative correlation between religiosity and conviction that evolutionary theory is scientific (F4). The more religious 15–16-year-olds were, the less convinced they were that the evolutionary theory is scientific. At the same time, more religious 13-14-year-old students were also more convinced that the evolutionary theory was scientific. This result is consistent with Mpeta et al. (2015) and Fiedler et al. (2024), who stated that the acceptance of evolution is also related to age, religiosity, and the time that has passed since the first learning about evolution.

A small negative correlation was found between attitudes toward biology (F3) in 13- to 14-year-olds and knowledge of human evolution. This is not surprising—despite a positive attitude toward biology, these students lacked knowledge of human evolution because they had not been formally taught this topic yet. Students 15 to 16 years old with more positive attitudes toward biology (F3) were more convinced that organisms evolved (F5). This could be explained by the findings of Prokop et al. (2007) and Kubiatko et al. (2017), who report that good teaching strategies and a positive teacher personality positively influence students' attitudes toward biology. It might be that students who were taught by such teachers were also more likely to accept the scientific facts presented by these teachers. The only correlation between two aspects of the acceptance of evolution was observed in 15- to 16-year-olds: the more the students accepted the theory of evolution (F2), the more they were convinced that it is scientific (F4).

Correlations calculated for the 13- to 14-year-olds were then compared with the correlations calculated for the 15- to 16-year-olds for statistical significance. Fourteen out of 15 correlations calculated differed significantly between these two groups (ps < .01). The results show that teaching evolution not only significantly improved the students' knowledge of human evolution but also caused changes in the acceptance of the evolutionary theory. This is in line with other studies that found positive correlations between knowledge and acceptance of evolution (Carter et al., 2015; Tavares & Bobrowski, 2018; Torkar & Šorgo, 2020; Salazar-Enriquez et al., 2023). There was no difference (p = .223) only regarding one correlation: in both groups, students who were more convinced that evolutionary theory is scientific (F4) were also more convinced that organisms evolve (F5). The fact that the 13- to 14-year-olds who had not learned evolution yet had the same convictions as the 15- to 16-year-olds was surprising, and it could be the consequence of contextual factors, such as the students' social surroundings (OECD, 2016; OECD, 2017; Šterman Ivančič & Mlekuž, 2023) or parental education level (Salazar-Enriquez et al., 2023).

A limitation of this study was its cross-sectional nature. A longitudinal study would have yielded more reliable results, but it would have been impossible to conduct due to logistical issues.

Conclusions

We can conclude that students in the ninth grade of elementary school improved their knowledge of human evolution. The progress was small to medium. The question is how to eliminate some important misconceptions our students have concerning Neanderthals, specifically that modern humans evolved from Neanderthals (rE1) and that modern humans and Neanderthals did not live on Earth at the same time (E36). The student's knowledge should also be improved on the following topics: modern humans evolved in Africa (E34) more than 15,000 years ago (rE33), they did not evolve from chimpanzees (rE35), and they lived at the same time as mammoths (E2). In these topics, attention should be given to teacher education programs in curricular renewal.

Author Contributions

Conceptualization, J.S.; methodology J.S.; formal analysis, J.S.; investigation, J.S.; data curation, J.S.; writing—original draft preparation, J.S.; writing—review, editing, and paper communication, J.S. All authors have read and agreed to the final version of the manuscript.

Conflicts of Interest

The author declares no conflict of interest.

References

Balgopal, M.M., 2014. Learning and intending to teach evolution: Concerns of pre-service biology teachers. Research in Science Education, 44, 27–52. Https://doi.org/10.1007/s11165-013-9371-0

Belin, C.M., Kisida, B., 2015. Science standards, science achievement, and attitudes about evolution. Educational policy, 29 (7), 1053–1075. https://doi.org/10.1177/0895904814550069

Beniermann, A., 2019. Evolution – von Akzeptanz und Zweifeln - Empirische Studien über Einstellungen zu Evolution und Bewusstsein [Evolution – of acceptance and doubts. Empirical studies on attitudes towards evolution and the evolution of the human mind]. Veröffentlichung der Dissertation, Wiesbaden: Springer Spektrum. https://doi.org/10.1007/978-3-658-24105-6

Bloom, P., Weisberg, D.S., 2007. Childhood origins of adult resistance to science. Science, 316, 996–997.

Borgerding, L.A., Klein, V.A., Ghoshm, R., Albert Eibel, A., 2015. Student teachers' approaches to teaching biological evolution. Journal of Science Teacher Education, 26:371-392. https://doi.org/10.1007/S1 DOI 10.1007/S1

Deniz, H., Anderson, E.S., 2016. Evolution acceptance and epistemological beliefs of college biology students. Journal of Research in Science Teaching, 54, 493–519. https://doi.org/10.1002/tea.21374

Carter, B.E., Infanti, L.M., Wiles, J.R., 2015. Boosting students' attitudes & knowledge about evolution sets them up for college success. The American Biology Teacher, 77 (2), 113–116. https://doi.org/10.1525/abt.2015.77.2.6

Cofre, H., Cuevas, E., Becerra, B., 2017. The relationship between biology teachers' understanding of the nature of science and the understanding and aceptance of the theory of evolution. International Journal of Science Education, 39 (16), 2243–2260. https://doi.org/10.1080/09500693.2017.1373410

da Silva Oliveira, G., Pellegrini, G., Araújo, L.A.L., Bizzo, N., 2022. Acceptance of evolution by high school students: Is religion the key factor? PloS ONE, 17 (9), e0273929-e0273929. https://doi.org/10.1371/journal.pone.0273929

Fiedler, D., Moormann, A., Beniermann, A., 2024. Using different acceptance measures: The interplay of evolution acceptance, evolution understanding, and religious belief among German pre-service biology teachers, secondary school students, and creationists. Science Education, 108 (1), 223–274. https://doi.org/10.1002/sce.21833

Field, A., 2009. Discovering statistics using SPSS. Sage Publications.

Flammer, L. (2006). The evolution solution: Teaching evolution without conflict. The American Biology Teacher, 68 (3), 1–7. https://doi.org/10.1894/0038-4909(2006)68[e1:TES]2.0.CO;2

Garibi, J.A., Antón, A., Villarroel, J.D., 2021. Information about human evolution: An analysis of news published in communication media in Spanish between 2015 and 2017. Publications, 9 (3), 28. https://doi.org/10.3390/publications9030028

Hartelt, T., Martens, H., Minkley, N., 2022. Teachers' ability to diagnose and deal with alternative student conceptions of evolution. Science Education, 106, 706–738. https://doi.org/10.1002/sce.2170

Hermann, R.S., 2013. On the legal issues of teaching evolution in public schools. The American Biology Teacher, 75 (8), 539–543. https://doi.org/10.1525/abt.2013.75.8.4

Kralj, L., Šalamon, T., Lukša, Ž., 2018. Usporedba znanja hrvatskih i slovenskih osnovnoškolaca te gimnazijalaca o evoluciji čovjeka. [Knowledge comparison of Croatian and Slovenian elementary high school students about human evolution]. Education Biologiae: Časopis Edukacije Biologije, 4, 31–34. https://doi.org/10.32633/eb.4.2

Kubiatko, M., Torkar, G., Rovnanova, L., 2017. The teacher as one of the factors influencing students' perception of biology as a school subject. CEPS Journal, 7(2), 127–140.

Mead, R., Hejmadi, M., Hurst, L.D., 2017. Teaching genetics prior to teaching evolution improves evolution understanding but not acceptance. PLoS Biology, 15 (5), e2002255-e2002255. https://doi.org/10.1371/journal.pbio.2002255

Mantelas, N., Mavrikaki, E., 2020. Religiosity and students' acceptance of evolution. International Journal of Science Education, 42 (18), 3071–3092. https://doi.org/10.1080/09500693.2020.1851066

Miller, J.D., Scott, E.S., Okamoto, S., 2006. Public Acceptance of Evolution. Science, 313 (5788), 765–766. http://dx.doi.org/10.1126/ science.1126746

Modell, H., Michael, J., Wenderoth, M.P., 2005. Helping the learner to learn: The role of uncovering misconceptions. The American Biology Teacher, 67, 20–26. http://dx.doi.org/10.1662/0002-7685(2005)067

Mpeta, M., de Villiers, J.J.R., Fraser, W.J., 2015. Secondary school learners' response to the teaching of evolution in Limpopo Province, South Africa. Journal of Biological Education, 49 (2), 150–164. https://doi.org/10.1080/00219266.2014.914555

OECD (2016). PISA 2015 Results (Volume I): Excellence in equity in education. OECD Publishing.

OECD (2017). PISA 2015 Results (Volume III): Students' well–being. OECD Publishing.

Pinxten, R., Vandervieren, E., Janssenswillen, P., 2020. Does integrating natural selection throughout upper secondary biology education result in a better understanding? A cross-national comparison between Flanders, Belgium and the Netherlands. International Journal of Science Education, 42 (10), 1609–1634. https://doi.org/10.1080/09500693.2020.1773005

Prokop, P., Prokop, M., Tunicliffe, S.D., 2007. Is biology boring? Student attitudes toward biology. Journal of Biological Education, 42(1), 36–39.

Rutledge, M.L., Sadler, K.C., 2007. Reliability of the measure of acceptance of the theory of evolution (MATE) instrument with university students. The American Biology Teacher, 69 (6), 332–335.

Salazar-Enriquez, G., Guzman-Sepulveda, J.R., Penaloza, G., 2023.Understanding and acceptance of the theory of evolution in high school students in Mexico. PloS ONE, 18 (2), e0278555-e0278555. https://doi.org/10.1371/journal.pone.0278555

Sjøberg, S., Schreiner, C., 2019. ROSE (The relevance of science education). The development, key findings and impacts of an international low cost comparative project. Final Report, Part 1 (of 2). University of Oslo.

Smith, M.U., 2010. Current status of research in teaching and learning evolution: II. Pedagogical issues. Science & Education, 19 (6), 539–571. https://doi.org/10.1007/s11191-009-9216-4

Stasinakis, P., Athanasiou, K., 2016. Investigating greek biology teachers' attitudes towards evolution teaching with respect to their pedagogical content knowledge: Suggestions for their professional development. Eurasia Journal of Mathematics, Science and Technology Education, 12 (6), 1605–1617. https://doi.org/10.12973/eurasia.2016.1249a

Strgar, J., 2010. Biological knowledge of Slovenian students in the living systems content area in PISA 2006. Acta Biologica Slovenica, 53 (2), 99–108. https://doi.org/10.14720/abs.53.2.15509

Šorgo, A, Usak, M, Kubiatko, M., Fančovičova, J., Prokop, P., Puhek. M., … Bahar, M., 2014. A cross–cultural study on freshmen's knowledge of genetics, evolution, and the nature of science. Journal of Baltic Science Education, 13 (1), 6–18. https://doi.org/10.33225/jbse/14.13.06

Šterman Ivančič, K., Mlekuž, A., 2023. Program mednarodne primerjave dosežkov učencev in učenk – PISA 2022. Nacionalno poročilo s primeri nalog iz matematike. [Program for international comparison of student achievement – PISA 2022. National report with examples of mathematics tasks]. Pedagoški inštitut, Ljubljana. https://www.pei.si/wp-content/uploads/2023/12/Porocilo_PISA22_FINAL.pdf

Štraus, M., Šterman Ivančič, K., Štigl, S., 2016. PISA 2015: naravoslovni, matematični in bralni dosežki slovenskih učenk in učencev v mednarodni primerjavi. [PISA 2015: Science, mathematics and reading achievements of Slovenian students in an international comparison]. Pedagoški inštitut, Ljubljana.

Tavares, G.M., Bobrowski, V.L., 2018. Integrative assessment of evolutionary theory acceptance and knowledge levels of biology undergraduate students from a Brazilian university. International Journal of Science Education, 40 (4), 442–458. https://doi.org/10.1080/09500693.2018.1429031

Torkar, G., Šorgo, A., 2020. Evolutionary content knowledge, religiosity and educational background of Slovene preschool and primary school pre–service teachers. Eurasia Journal of Mathematics, Science and Technology Education, 16 (7), em1855. https://doi.org/ 10.2307/4451708

Učni načrt. Program osnovna šola. Biologija. [Curriculum. Primary School Program. Biology]. 2011. Ministrstvo za šolstvo in šport; Zavod RS za šolstvo.

Učni načrt. Program osnovna šola. Zgodovina. [Curriculum. Primary School Program. History]. 2011. Ministrstvo za šolstvo in šport; Zavod RS za šolstvo.

Vaughn, A.R., Robbins, J.R., 2017. Preparing pre-service K-8 teachers for the public school: Improving evolution attitudes, misconceptions, and legal confusion. Journal of College Science Teaching, 47 (2), 7–15. https://doi.org/10.2505/4/jcst17_047_02_7

Vesel, B., 1977. Preobrazba filogenetskega koncepta v biološki koncept pouka biologije v gimnazijah SRS. [The transformation of the phylogenetic concept of biology lessons in SRS grammar schools into the biological concept]. [Doctoral dissertation, University of Ljubljana, Biotechnical Faculty].

Wescott, D.J., Cunningham, D.L., 2005. Recognizing student misconceptions about science and evolution. MountainRise, 2, 1–8.

Yates, T.B., Marek, E.A., 2014. Teachers teaching misconceptions: A study of factors contributing to high school biology students' acquisition of biological evolution-related misconceptions. Evolution: Education and Outreach, 7 (1), 7. https://doi.org/10.1186/s12052-014-0007-2

ABS

Original Research

Unveiling the antibacterial potential of latex-derived phytocomponents from *Calotropis gigantea* targeting bacterial Penicillin-binding proteins: chemical profiling, *in silico* docking, and in vitro lab validation

Arun Dev Sharma [1*], Amrita Chauhan [1]

Abstract

Uncontrolled use of currently accessible medicines has caused antibiotic resistance in bacteria worldwide. This work aimed to undertake molecular docking and chemical profiling of bioactive components extracted from *Calotropis gigantea* latex, with wet lab confirmation to follow. Gas chromatography was used to analyze the phytochemical profile. The docking tool, Cb-dock2, was used for in-silico analysis. G+ and G- bacterial strains were utilized to confirm the wet lab results. Chemical profiling revealed 18 peaks, and some major bioactive compounds were like phytol, 4-methyl-2- phenylindole and α-tocospiro A. Docking analysis revealed strong interaction against bacterial Penicillin-binding Protein (PBP1a) with docking scores ranging from -5.2 to -7.9 (Vina score). The PBP-Ligand complex has Van der Waals, hydrogen bond, pi-cation, and alkyl bonding interactions, according to the results of the interaction investigations. Calotropis latex successfully suppressed the development of bacterial strains (G+ and G-) with inhibition zones ranging from 1.1 cm to 2.2 cm, as demonstrated by in-vitro tests, indicating their potential as antibacterial medications. The study found that latex-based bioactive compounds have the potential to be used in the pharmaceutical and biotechnology sectors by facilitating the creation of structural alterations that lead to stronger medications. Therefore, this latex-derived compound may open a new pipeline into the development of multi-target antibiotics against a broad-spectrum multidrug-resistant G- bacterium.

Keywords

Penicillin-binding protein, calotropis latex, in-silico studies, anti-bacterial

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Razkritje antibakterijskega potenciala iz lateksa pridobljenih fitokomponent iz *Calotropis gigantea*, usmerjenih proti bakterijskim proteinom, ki vežejo penicilin: kemijsko profiliranje, *in silico* docking in in vitro potrditev v laboratoriju

Izvleček

Nenadzorovana uporaba trenutno dostopnih zdravil je povzročila razvoj odpornosti bakterij proti antibiotikom po vsem svetu. Namen tega dela je bil izvesti analizo molekularnega prileganja in kemijsko profiliranje bioaktivnih sestavin, pridobljenih iz lateksa *Calotropis gigantea*, čemur bo sledila potrditev z eksperimenti. Za analizo fitokemičnega profila je bila uporabljena plinska kromatografija. Za *in silico* analizo je bilo uporabljeno orodje Cb-dock2. Za potrditev rezultatov v laboratoriju so bili uporabljeni sevi G+ in G- bakterij. Kemijsko profiliranje je pokazalo 18 vrhov, nekatere glavne bioaktivne spojine pa so bile fitol, 4-metil-2- fenilindol in α-tokospiro A. Analiza prileganja je pokazala močno interakcijo z bakterijskim proteinom, ki veže penicilin (PBP1a), z rezultati dokiranja od -5,2 do -7,9 (ocena Vina). V kompleksu PBP-ligand so glede na rezultate preiskav interakcij prisotne Van der Waalsove vezi, vodikova vez, pi-kation in alkilna vez. Lateks kalotropisa je uspešno zaviral razvoj bakterijskih sevov (G+ in G-) z inhibicijskimi conami od 1,1 cm do 2,2 cm, kot so pokazali *in vitro* testi, kar kaže na njihov potencialno uporabnost kot antibakterijska zdravila. Študija je pokazala, da imajo bioaktivne spojine na iz lateksa *Calotropis gigantea* potencial za uporabo v farmacevtskem in biotehnološkem sektorju, saj omogočajo ustvarjanje strukturnih sprememb, ki vodijo k močnejšim zdravilom, zato lahko ta spojina, pridobljena iz lateksa, odpre novo pot za razvoj multitarčnih antibiotikov proti širokemu spektru G- bakterij z multiplo rezistenco.

Ključne besede

penicillin vezajoč protein, mleček kalotropisa, *in silico* študije, protibakterijsko delovanje

Introduction

Since they were developed, antibiotics have significantly benefited human health and the battle against bacterial illnesses. However, in recent years, antibiotic abuse has resulted in medicine-resistance-created antibiotic-resistant microorganisms (ARMs), which present serious health risks to individuals. Therefore, the researchers' current goal is to develop innovative, safe drugs (Mir et al., 2022). The emergence of antibiotic resistance has made microbes one of the greatest risks to human health (Harris et al., 2022). G-bacteria are unique to hospitals and communities; they have developed resistance to all currently available antibiotics.

β-lactam antibiotics have been targeting membrane-bound macromolecules involved in forming cell walls, known as penicillin-binding proteins (PBPs), for over 70 years. Numerous PBPs are found in bacteria, and some are starting to show their special roles in the cell division cycle (Straume et al., 2020). Peptidoglycan is depleted and may cause cell death when β-lactam antibiotics block transpeptidation or carboxypeptidation activities (Chan et al., 2016). This powerful mechanism, which has made penicillin and its analogues the most extensively used antibiotics globally for all infectious illnesses over the past 70 years, is under pressure due to the emergence of penicillin and its analogue resistance. This highlights the necessity for cutting-edge, all-natural antibiotic remedies. Additionally, it has been observed that the naturally occurring substance moenomycin inhibits the actions of glycosyltransferases, which cause bacterial cells to die by breaking down peptidoglycan (Masters et al., 2020). Glycan chain polymerization (transglycosylation) and glycan chain cross-linking (transpeptidation) are catalyzed by PBPs. Certain PBPs hydrolyze the last D-alanine of the stem pentapeptide (DD-carboxypeptidation) or the peptide link between the two glycan chains (endopeptidation), according to Straume et al. (2020). One of the most promising antibiotic targets is the mechanism involved in bacterial cell wall production. A comparable macromolecular framework is absent from human metabolism, but many proteins, including PBPs, are

involved in the metabolism of bacteria, which is essential to life. These two prerequisites must be met in order to produce antibiotics. Furthermore, it has been noted that the naturally occurring compound moenomycin suppresses the activities of glycosyltransferases, which degrade peptidoglycan and kill bacterial cells (Masters et al., 2020). PBPs are thought to be great candidates for the creation of bacterial inhibitors due to their important role. The function of the PBP protein can be blocked to prevent bacterial replication. No PBP with a similar cleavage specificity has been found in humans; hence, the inhibitors are unlikely to be considered dangerous.

Because they are safer, more ecologically friendly, and have no unfavourable side effects, conventional medical procedures are therefore improving and growing in popularity (Mir et al., 2022). People still favour natural herbal remedies over synthetic ones despite the many advantages that synthetic drugs provide. Because they include multiple essential botanical components in various plant sections, medicinal plants are unique in that they may treat and cure a wide range of human health concerns (Salm et al., 2023; Chaachouay and Zidane, 2024). Natural plant products, like plant-based latex, are rich in bioactive compounds that vary in both biology and molecular structure, making them important tools for the creation of new drugs (Nasim et al., 2022).

Calotropis gigantea is commonly known as the giant milkweed and belongs to the Asclepiadaceae family (Wijeweera et al., 2020). In traditional Ayurvedic medicine, *Calotropis gigantea* is called "Sweta Arka" and "Rakha Arka", respectively, and it is designated an ethnomedical useful plant (Julius et al., 2021). It is a perennial shrub native to diverse regions encompassing tropical Africa and Asia (Wijeweera et al., 2020). This species is widely distributed across roadsides, railway tracks and deserted land pockets and can be found in countries such as Bangladesh, Burma, China, India, Indonesia, Malaysia, Pakistan, the Philippines, Thailand, Nepal, and Sri Lanka, where it holds significant economic importance (Gyawali et al., 2020). *Calotropis gigantea* is commonly known as milkweeds because of the latex they produce (Wijeweera et al., 2020). Plants produce white latex when sliced or broken. Several plant parts, including the flowers, stem, and leaves, can be used to extract latex (Timilsina et al., 2020). This white latex is loaded with several bioactive compounds. However, due to the biologically active substances such as alkaloids, flavonoids, terpenoids, glycosides, phenolics, tannins, and saponins that it contains (Kumar et al., 2021), plant latex has made a significant contribution to medicine. Moreover, some ethnomedicinal applications of *Calotropis* plant parts include pesticide, anti-inflammatory, anti-fertility, antiulcer, fungicide, antiseptic, insecticide, anti-diabetic, and anti-helminthic activity (Wadhwani et al., 2021). Nevertheless, very little research on antioxidant bioactive compounds has been done on the latex of such a neglected plant species, even though this plant is widely utilized by the local communities as food ethnomedicine and is thought to be very nutritious. Consequently, in order to support *Calotropis gigantea* applications in traditional medicine, research into the latex secondary metabolite compositions, particularly its richness in phytonutrients, is still needed. Through the use of computational approaches to drug discovery, sophisticated methodologies that may be applied to screen drugs derived from phytochemicals found in a variety of medicinal plants have been established. Computational prediction models are critical to guide the technique selection process for drug and technology development. Furthermore, they have been used in the in-silico prediction of pharmacokinetic, pharmacological, and toxicological parameters, per Loza-Mejía et al. (2018). Molecular docking has become a practical and affordable approach for drug discovery and evaluation. As per the findings of Lee et al. (2019), this technique provides a valuable understanding of drug-receptor interactions and may be applied to predict the orientation of a potential medication upon binding to a target protein. However, because of its intricacy, the precise mechanism behind *Calotropis gigantea* latex's antibacterial action is ultimately unclear. We've spoken about how we think Calotropis gigantea latex's several bioactive ingredients may help reduce the threat posed by both G+ and G- bacteria that are resistant to multiple drugs. Consequently, further research is needed to determine the possible medicinal advantages and workable modes of action of *Calotropis gigantea* latex. The objective of this study was to perform phytochemical screening and in-silico docking of the most common compounds present in *Calotropis gigantea* latex against target proteins involved in the bacterial life cycle. Additionally, it will offer new insights into future projections for figuring out the dose of crucial antibacterial drugs. This will increase our knowledge of the potential therapeutic use of the latex from the *Calotropis gigantea* plant. These techniques seek to offer a thorough comprehension of the characteristics and chemical makeup of *Calotropis gigantea* latex, as well as its potential therapeutic effects.

Material and Methods

Collection of samples

Wildly growing *Calotropis gigantea* (Supplementary Fig. 1) plant samples were collected from roadside locations close to the study area (31.30 latitudes North and 75.60 longitude East). The plant was verified by the botany department, and BT103 was noted on the voucher. This city experiences long, hot summers and chilly winters due to its humid subtropical climate. The study was carried out between January and May of 2024.

Preparation of extracts

The latex (about 30 mL) was collected from the *Calotropis gigantea* plant by breaking the stems of the plant with hands and then squeezing the broken stems to collect the latex from them into the Eppendorf tubes and stored at 4 °C for further use.

GC-FID analysis of latex

Chemical profiling was done using a gas chromatography flame ionization detector (GC-FID) system (Chemtron 2045) for the study of bioactive chemicals (Sharma and Kaur, 2022a). To identify and measure the bioactive chemicals contained in *Calotropis gigantea* latex, it was mixed with double volumes of isopropyl alcohol/water (8:2 v/v). Shaking the mixture by hand, vortexing it, and letting it lie overnight at room temperature were the next steps before centrifuging it for 15 minutes at 4 °C at 10,000 rpm. After that, the supernatant was collected and subjected to GC-FID examination. A 0.2 µl injection volume of extract was used. A 2-meter-long stainless-steel column fitted with a 10% OV-17 load on an 80–100% mesh Chromosorb W (HP) filter was provided by GC. The carrier gas was nitrogen gas at a rate of 30 mL per minute. The temperature of the injector and detector was maintained at 200°C and 250°C, respectively. A 0.2µl sample volume was injected. The ramping temperature settings for the oven were 110°C initially, then 200°C at a pace of 2°C per minute. Bioactive compounds were identified by comparing the relative retention duration with published data or standards (Adam, 2007).

Ligands Preparation

For in-silico docking studies, the predominant phytochemicals in *Calotropis gigantea* latex (Phytol, 4-Methyl-2-phenylindole and α-Tocospiro A) were chosen were ligands for seven bacterial Penicillin-binding protein (PBP1a). The following link was used to retrieve SMILES of ligands: https://pubchem.ncbi.nlm.nig.gov/. The 3-D structures of ligands were generated using UCSF-chimera after SMILES were obtained from the NCBI-PubChem database.

Protein preparation

Targets bacterial penicillin-binding protein (PBP1a) was the protein involved in the bacterial life cycle and was hence selected as a key target for docking. The RCSB database (https://www.rcsb.org/) was used to retrieve the target macromolecule (PDB IDs: PBP1a:3UDF). The PBP1a structure lacks water molecules and natural inhibitors before docking research. The dock-prep function in the Chimera program (chimaera tool 1.14) was used to lower energy. The optimization process of dock preparation takes atomic structure, bond length, and charge anomalies into consideration.

Molecular Docking

A molecular docking study was performed on the selected phytocompounds using the CB-Dock2 application (https://cadd.labshare.cn/cb-dock2/php/index.php). The protein receptor (PBP1a) and ligand molecules were then sent to the CB-Dock2 service in pdb file format. A .pdb file with the ideal docked structure was generated and downloaded.

In-vitro Anti-bacterial Activity

The antimicrobial activity of calotropis latex was assessed using the agar disc diffusion method against four test organisms: G+ *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (MTCC 121), and G- *Escherichia coli* (MTCC 40), *Pseudomonas aeruginosa* (MTCC 424). The microorganisms originate from IMTECH, the Institute of Microbial Technology in Chandigarh. *Calotropis* latex (volume: 5-150µl) was impregnated onto sterilized paper discs (5 mm diameter). New inoculums were created using 12-hour-old cultures for each microbial strain. After swabbing the LB-Agar plates with bacterial suspension, they were allowed to dry for thirty minutes. Next, the calotropis latex-impregnated paper discs

were positioned in the middle of the petri plates. The plates underwent incubation for 24 hours at 37°C after being kept at room temperature for a period of 20 minutes to enable the calotropis latex out of the disc to dissociate into the liquid. Streptomycin (10µg/disc) served as the positive control. After incubation, a zone of inhibition encircling the disc may be observed using a transilluminator; this radius represents the antibacterial activity of calotropis latex.

Statistical Analysis

The average ± standard deviation is displayed for each study; all investigations were carried out in triplicate using at least three different trials unless otherwise stated. ANOVA (analysis of variance) was utilized with GraphPad Prism v. 6.0 (GraphPad Software, Inc., San Diego, CA, USA) to identify significant differences. Tukey's multiple comparisons test was employed to evaluate mean differences between groups. Variations were considered significant when $p < 0.05$.

Results and Discussion

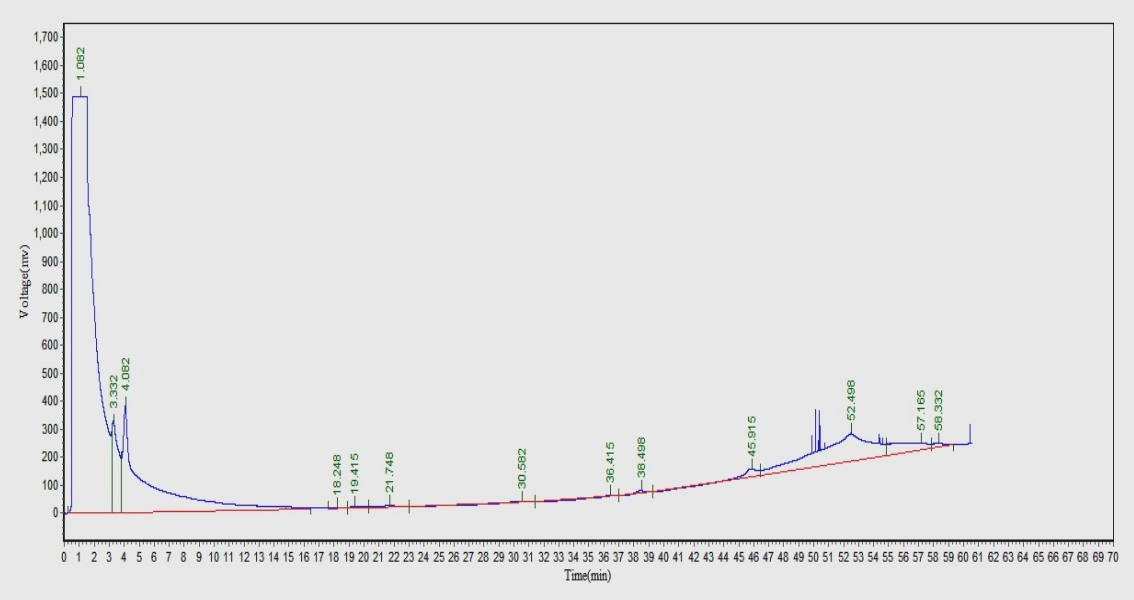
Gas chromatography analysis

Phytochemical profiling is the qualitative test based on colour formations and/or precipitates of herbal formulations as indicators of the presence of secondary metabolites in plant material (Bouzidi et al., 2023). Many contemporary analytical techniques have been developed, and they are crucial in determining the active ingredients, medicinal properties, pharmacological properties, and other details of a substance's application in the fields of biochemical, pharmaceutical, and clinical research. GC-FID phytochemical analysis is one of the contemporary methods for identifying and isolating phytoconstituents. One modern technique for identifying and isolating phytoconstituents in plants is GC-FID (Bouzidi et al., 2023; Nkwocha et al., 2023; Onyeike et al., 2023).

The typical chromatogram depicting phytochemical compounds present in calotropis latex received by GC-FID is evident in Figure 1. Additionally provided are the noted peaks and the duration of their retention. GC-FID analysis of latex extracted from *Calotropis gigantea* led to the identification of 13 compounds. Oxygenated diterpenes, i.e. Phytol, with other minor components, were found to be the most dominant phytocompounds at 64%. Acyclic hydrogenated diterpene alcohol, or phytol, is the main component and precursor to synthetic forms of vitamins K and E. It is also directed as phytosol or floral alcohol (Gutbrod et al.,2021). Phytol is used in the production of fragrances. According to Islam et al. (2018), it is also used commercially in shampoos, toilet soaps, detergents, and cosmetics. Additionally, some cannabis distillates use it as a flavouring or dilution. The microscopic peaks could be caused by the bioactive compounds present in trace amounts. The latex composition turned out to be different from previous investigations. Prior research has likewise confirmed the existence of both main and minor elements, albeit with different compositions. For example, in our investigation, phytol accounted for 24% of the total component; in contrast, Al-Rowaily et al. (2020) found that the phytol content was 8.73%. The current findings were entirely consistent with the *C. procera* EO chemical profile that was published and obtained from the Egyptian delta, where oxygenated diterpene phytol was found in amounts of 38% (Wahba and Khalid, 2018). These findings were consistent with those published for other Egyptian *C. procera* organs, where the primary chemicals were identified as diterpenes, specifically E-phytol. However, the profile of Indian *C. gigantea* (Singh and Javed, 2013) showed no diterpenes at all, which was at odds with the current results. Overall, the variance in the metabolites' production, including that of latex, may be the cause of the considerable disparity in chemical components. Environmental and climatic factors such as temperature, altitude, seasonality, plant age and water availability and soil conditions such as weather, extraction technique, soil moisture, harvest season, the distance between plants, harvest time, drying method, storage conditions, conditions, and genotype, all have a direct impact on the biosynthesis of latex components (Borges et al., 2018, Amin et al., 2019).

Molecular Docking studies

In-silico study was conducted by targeting PBP 1(Penicillin-binding protein), which is essential for the final steps of peptidoglycan (PG) bacterial cell wall biosynthesis (Galinier et al., 2023). The primary structural element of the bacterial cell wall, PG is necessary to preserve the form, integrity, and survival of the cell. Owing to transpeptidase activity, PBP1 is involved in connecting the peptide side chains of developing glycan strands in cell wall synthesis, thus



S. No	Ret. Time (RT)	Bioactive Compound	Conc. (%)
1	1.082	Phytol	64.4103
2	3.332	Butanoic acid, 2[(phenyl methoxy)imino] trimethylsilyl ester	4.4500
3	4.082	4-Methyl-2- phenylindole	16.2854
4	18.248	Cyclohexanol,3-methyl	0.0594
5	19.415	Ergost-5-en-3-ol	0.0841
6	21.748	β-Cyclocitral	0.1554
7	30.582	Heptadecyl acetate	0.1362
8	36.415	α-amryin	0.1346
9	38.498	Lup-20(29)-en-3-ol acetate	0.1547
10	45.915	Guanidine nitrate	0.4865
11	52.498	α-Tocospiro A(alpha-tocopheroids)	10.9942
12	57.165	Ursa-12-en-3β-yl pentanoate	2.3091
13	58.332	Astilbin	0.3400

Figure 1. Chemical profile and phytocompounds identified calotropis latex. Green font indicates major compounds used for docking analysis.

Slika 1. Kemični profil in identificirane fitokomponente lateksa kalotropisa. Črke v zeleni barvi označujejo glavne spojine, uporabljene za analizo prileganja.

being selected as the ideal target for docking studies. The bioactive compounds with the most potential for inhibition are those with the lowest vina score with their targets. According to Agu et al., 2023, molecular docking is an efficient computational method applied here to anticipate and determine the potential binding mode that emerges between a chemical and a specific target protein or receptor. Penicillin-binding proteins (PBP1) were used in this study's molecular docking assays, with the most prevalent phytocompounds found in calotropis latex. The docking score of every bioactive molecule against the PBP1 and other pertinent data are displayed in Table 1.

Table 1. Molecular docking of α-amylase with the bioactive component of latex derived as ligand.

Tabela 1. Molekularno prileganje α-amilaze z bioaktivno komponento lateksa kot ligandom.

Ligand	Vina score	Cavity volume (Å^3)	Center (x, y, z)	Docking size (x, y, z)	Contact residues
Phytol	-5.2	2357	93, 48, -1	27, 27, 27	Chain A: LEU64 ALA65 GLU67 ASP68 SER69 SER70 PHE71 ASN134 TRP205 ARG209 GLN212 LEU213 Chain B: GLN506 MET509 ASP510 PHE511 GLY512 LEU513 GLN514 GLU515 ASP516 LEU532 ILE534 GLN535 THR538 ARG549 VAL550 GLN551 PRO552
4-Methyl-2- phenylindole	-7.4	2357	93, 48, -1	29, 20, 32	Chain A: ALA66 GLU67 ASP68 SER69 SER70 PHE71 ASN134 LYS185 ARG202 TRP205 ARG209 GLN212 LEU213 Chain B: LYS255 LEU426 GLN427 TRP429 GLN506 MET509 ASP510 GLY512 LEU513 GLN514 GLU515 ASP516 LEU532 ILE534 GLN535 THR538 ARG549 GLN551 PRO552
α-Tocospiro A	-7.9	2357	93, 48, -1	29, 29, 29	Chain A: ALA66 GLU67 ASP68 SER69 SER70 PHE71 ASN134 LEU135 LYS185 ALA186 TYR190 ARG202 TRP205 ARG209 GLN212 LEU213 GLY214 TYR215 Chain B: GLU247 ARG250 SER251 GLU252 VAL254 LYS255 GLU259 LYS421 PHE422 ASN423 LEU426 GLN427 TRP429 MET509 ASP510 GLY512 LEU513 GLN514 GLU515 LEU532 ILE534 GLN535 THR538 ARG549 GLN551 PRO552 GLU572 GLN631 ILE633
Phytol	-5.2	2357	93, 48, -1	27, 27, 27	Chain A: LEU64 ALA65 GLU67 ASP68 SER69 SER70 PHE71 ASN134 TRP205 ARG209 GLN212 LEU213 Chain B: GLN506 MET509 ASP510 PHE511 GLY512 LEU513 GLN514 GLU515 ASP516 LEU532 ILE534 GLN535 THR538 ARG549 VAL550 GLN551 PRO552
4-Methyl-2- phenylindole	-7.4	2357	93, 48, -1	29, 20, 32	Chain A: ALA66 GLU67 ASP68 SER69 SER70 PHE71 ASN134 LYS185 ARG202 TRP205 ARG209 GLN212 LEU213 Chain B: LYS255 LEU426 GLN427 TRP429 GLN506 MET509 ASP510 GLY512 LEU513 GLN514 GLU515 ASP516 LEU532 ILE534 GLN535 THR538 ARG549 GLN551 PRO552
α-Tocospiro A	-7.9	2357	93, 48, -1	29, 29, 29	Chain A: ALA66 GLU67 ASP68 SER69 SER70 PHE71 ASN134 LEU135 LYS185 ALA186 TYR190 ARG202 TRP205 ARG209 GLN212 LEU213 GLY214 TYR215 Chain B: GLU247 ARG250 SER251 GLU252 VAL254 LYS255 GLU259 LYS421 PHE422 ASN423 LEU426 GLN427 TRP429 MET509 ASP510 GLY512 LEU513 GLN514 GLU515 LEU532 ILE534 GLN535 THR538 ARG549 GLN551 PRO552 GLU572 GLN631 ILE633

*Green font indicates common residues among ligands involved in interactions.

Figure 2 shows 3D models of the PBP1 complex with affinities ligands and their interactions. With vina scores ranging from -5.2 to -7.9, the three common phytochemicals found in the latex were demonstrated to have binding potentials. α-Tocospiro-A exhibited the most efficient binding of all the bioactive components, with a docking score of -7.9, followed by 4-Methyl-2- phenylindole (vina core -7.4) and phytol (vina score -5.2). The ligand's affinity for the receptor is determined by its ability to create hydrogen bonds or hydrophobic interactions with the receptor protein's active site residues during docking (Sharma and Kaur, 2022b). It was observed that almost all phytochemicals interacted with the PBP1a receptor via Van der Waal, alkyl, and Pi-alkyl interactions (Table 1). Notable all phytocompounds involved some common residues located at A and B chains to interact with the PBP1a receptor, as shown in Table 1. Antibacterial agents have a wide range of targets at which they can prevent the formation of cell walls. For many years, these systems have been regarded as significant targets for antimicrobial agents (Bruning et al., 2011). It has been discovered that natural substances prevent PBP from forming cell walls. The transpeptidase action of PBP is attributed to the C-terminal module (Contreras-Martel et al. 2017). Studies have demonstrated that therapies that mimic the structure

of the D-peptide moiety, D-body, such as β-lactam antibiotics, can obstruct the processes of transpeptidation or carboxypeptidation, which depletes peptidoglycan and may even result in cell death (Da-Costa et al. in 2018). This results in decreased cell wall integrity and the inability to transfer peptidoglycan (Moon et al., 2018). The results of Sripathi and Ravi (2017) reported that molecular docking analyses to determine the antibacterial efficiency of the most active components of Plectranthus hadiensis essential oil against PBPs are consistent with our findings. Additionally, molecular docking investigations of Pogostemon cablin essential oil components against PBPs were published by Yang et al. (2013). The study's findings suggest that plant-based metabolites might be the primary source of antibacterial chemicals. Based on the aforementioned observations, it can be inferred that phytocompounds exhibited a strong affinity for PBP1a upon ligand binding. Consequently, it was postulated that PBP1a alteration results in a modification of the bacterial cell wall and enzyme conformation. By stopping bacterial viability, all of these occurrences lessen the bacteria's capacity to invade the host cell. Previous research has also shown through in-silico results that multi-pharmacological drugs have antibacterial properties that can regulate the proliferation of microbial strains (Lima et al., 2019).

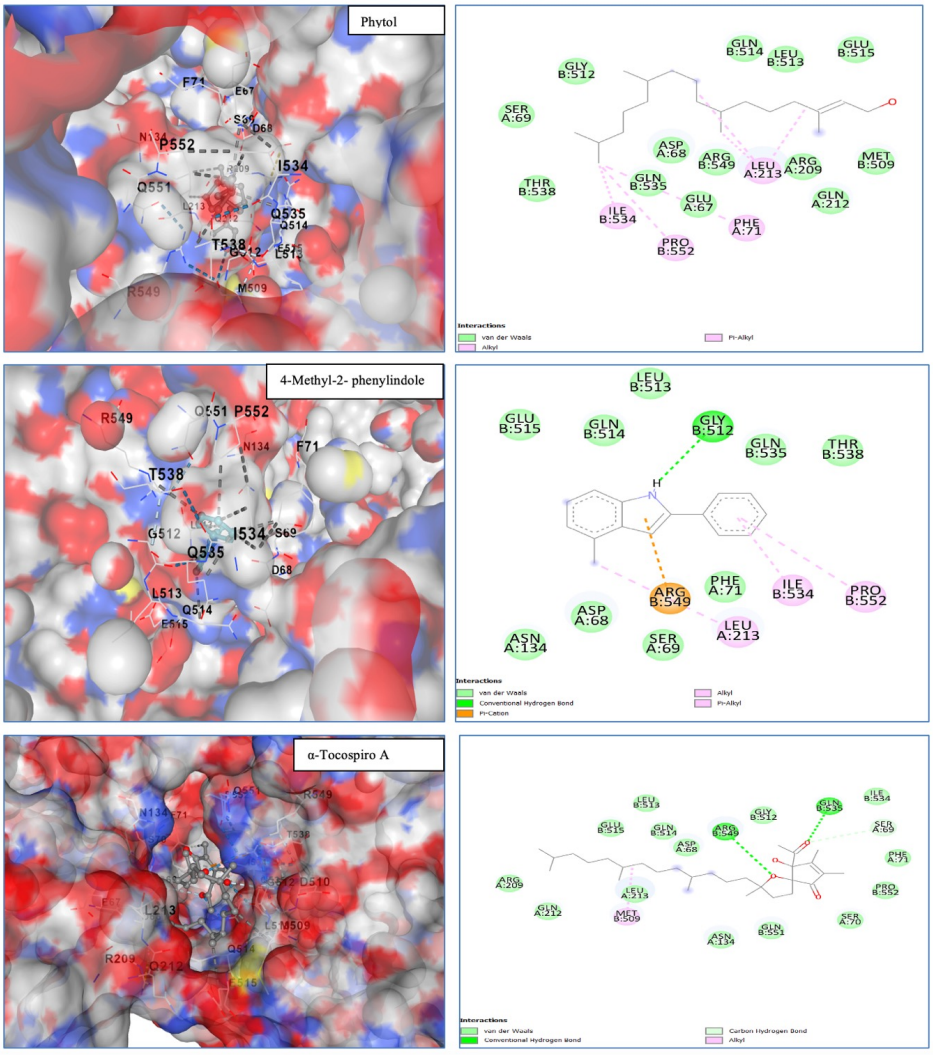


Figure 2. 2D models and 3D interactions of phytocompounds with PBP1a receptor.

Slika 2. 2D-modeli in 3D-interakcije fitokomponente z receptorjem PBP1a.

Antibacterial potential of latex

The wet lab experiment study was designed to assess the anti-bacterial potential of calotropis latex against four bacterial strains to validate the in-silico findings. According to the current analysis, calotropis latex exhibited potent antibacterial activity comparable to synthetic antibacterial drugs. Table 2 and Supplementary Figure 1-5 present the findings.

At 150µl, calotropis latex showed very strong antibacterial activity against P.aeruginosa (MTCC 424 G-) and Staphylococcus aureus (MTCC 3160 G+) with inhibition zone (IZ) of 2.2cm and 1.6cm, respectively. *E.coli* (MTCC 40 G-) and *B.subtilis* (MTCC 121 G+) exhibited moderate inhibitory activity with IZ values of 1.1 cm and 1.2 cm, respectively. The potent antibacterial properties of calotropis latex could be attributed to the presence of major and minor bioactive components in toto that suppressed partners such as microbial adhesions, cell wall envelop proteins, and non-specific interactions with carbohydrates, or affected the inhibition of hydrolytic enzymes (proteases) (Saddiq et al. (2022). Calotropis latex may have multiple targets or a single target, contributing to its varying antibacterial activity against different bacterial strains. Additionally, prior research has shown that the synergistic effects of major and minor components, rather than the high concentration of a single chemical compound, are often responsible for anti-microbial activity (Vaou et al., 2022). Previous studies have shown that anti-microbial activity can occasionally be attributed to the combined effects of major and minor chemical components as opposed to a high concentration of a single main chemical molecule (Vaou et al., 2022).

Table 2. Anti-bacterial activity of calotropis latex of different microbial cultures.

Tabela 2. Protibakterijsko delovanje lateksa kalotropisa na različne mikrobne kulture.

Microbial culture	Volume (µl)	Inhibition zone (cm)
Escherichia coli MTCC 40	NC	-
	PC	2±0.02a
	5	NI
	20	0.7±0.03c
	50	0.9±0.03c
	150	1.1±0.04c
Bacillus subtilis MTCC121	NC	-
	PC	1.5±0.01d
	5	NI
	20	0.6±0.03c
	50	0.9±0.02c
	150	1.2±0.02c
Pseudomonas aeruginosa MTCC424	NC	-
	PC	1.6±0.02d
	5	NI
	20	1±0.02c
	50	1.3±0.04d
	150	2.2±0.02e
Staphylococcus aureus MTCC3160	NC	-
	PC	1.7±0.01d
	5	NI
	20	0.5±0.02c
	50	1.0±0.03c
	150	1.6±0.04d

Note: PC: Positive Control, NC: negative control (Streptomycin 10 µg); NI: no inhibition. Results are expressed as Mean ± SD of three determinations (n = 3). Mean values with different letters (a-d) across the columns are considered significantly different at $p < 0.05$.

Nejhad et al.'s study from 2023 also mentioned the antibacterial activity of an aqueous extract of *Calotropis procera* leaves against *E. coli*, but IZ was only 14.1 mm. One significant finding from our study's CEO was that G-bacteria were highly toxic to antibiotics. This is notable because earlier studies have suggested that G-bacteria's thicker cell walls allow them to express resistance to drugs, and antibiotics in particular, making them more resistant than G+ bacteria (Saxena et al., 2023). The chemical components found in the plant combination are often responsible for the antibacterial action of a plant extract. Rather than coming from the presence of a single major active element, a medicinal plant's optimum effectiveness may come from the combination of multiple diverse ingredients (Nejhad et al., 2023). The primary cause of the variations in antibacterial activity is the plant's varied concentration of secondary metabolites, which may be impacted by elements including species and extraction techniques. Compared to G+ bacteria, which have a single cell wall structure, G- bacteria enclose a multilayered cell wall structure, increasing their resistance to antimicrobial agents (Nooshkam et al., 2019; Shahidi et al., 2020). In recent times, there has been a growing significance placed on the antibacterial properties of plant flavonoids against various microbial species. Plant extracts typically have antimicrobial properties because they contain phenolic compounds with free hydroxyl groups, like flavonoids. These substances can disrupt bacterial membrane permeability, change cellular peptidoglycan, bind to the active site of enzymes, form hydrogen bonds with enzymes, and disrupt soluble and extracellular proteins, glutamate, and phosphate of bacteria, among other mechanisms (Shahidi et al., 2020).

Conclusion

Antibiotic resistance to both G+ and G- bacteria is now present in human populations and might endanger health around the globe. PBP's are currently the primary focus of infection. The goal of this work was to examine the bioactive components of calotropis latex that may be utilized to block bacterial infection pathways. Effective docking of all major phytocompounds with PBP1 and antibacterial activity has been demonstrated by docking and in vitro investigations. Therefore, we suggest that calotropis latex, which is included in herbal medications, might be a viable treatment choice and function as a bacterial PBP inhibitor. To make space for these compounds in drug development, more research utilizing in vitro and in vivo models should be carried out to validate these compounds.

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Author's contributions

Conceptualization, ADS; methodology, AC.; software, AC; validation, ADS.; formal analysis, ADS and AC; investigation, ADS; writing—original draft preparation, ADS; writing—review and editing, ADS; visualization, AC; supervision, ADS; project administration, ADS; funding acquisition, ADS. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References

Agu, P.C., Afiukwa, C.A., Orji, O.U., Ezeh, E.M., Ofoke, I.H., Ogbu, C.O., Ugwuja, E.I., Aja, P.M., 2023. Molecular docking as a tool for the discovery of molecular targets of nutraceuticals in diseases management. Scientific Reports, 13, 13398. DOI: 10.1038/s41598-023-40160-2.

Nejhad, A., Alizadeh Behbahani, B., Hojjati, M., Vasiee, A., Mehrnia, M.A., 2023. Identification of phytochemical, antioxidant, anticancer and antimicrobial potential of Calotropis procera leaf aqueous extract. Scientific Reports, 13, 14716. DOI: 10.1038/s41598-023-42086-1.

Al-Rowaily, S.L., Abd-ElGawad, A.M., Assaeed, A.M., Elgamal, A.M., Gendy, A.E.G.E., Mohamed, T.A., Dar, B.A., Mohamed, T.K., Elshamy, A.I., 2020. Essential Oil of Calotropis procera: Comparative Chemical Profiles, antimicrobial Activity, and Allelopathic Potential on Weeds. Molecules, 25, 5203. DOI: 10.3390/molecules25215203, PubMed: 33182287.

Amin, S.M., Hassan, H.M., El Gendy, A.E.N.G., El-Beih, A.A., Mohamed, T.A., Elshamy, A.I., Bader, A., Shams, K.A., Mohammed, R., Hegazy, M.E.F., 2019. Comparative chemical study and antimicrobial activity of essential oils of three *Artemisia* species from Egypt and Saudi Arabia. Flavour and Fragrance Journal, 34, 450–459. DOI: 10.1002/ffj.3525.

Borges, R.S., Lima, E.S., Keita, H., Ferreira, I.M., Fernandes, C.P., Cruz, R.A.S., Duarte, J.L., Velázquez-Moyado, J., Ortiz, B.L.S., Castro, A.N., Ferreira, J.V., da Silva Hage-Melim, L.I., Carvalho, J.C.T., 2018. Anti-inflammatory and antialgic actions of a nanoemulsion of *Rosmarinus officinalis* L. essential oil and a molecular docking study of its major chemical constituents. Inflammopharmacology, 26, 183–195. DOI: 10.1007/s10787-017-0374-8.

Bouzidi, A., Azizi, A., Messaoudi, O., Abderrezzak, K., Vidari, G., Hellal, A.N., Patel, C.N., 2023. Phytochemical analysis, biological activities of methanolic extracts and an isolated flavonoid from Tunisian *Limoniastrum monopetalum* (L.) Boiss: An in vitro and in silico investigations. Scientific Reports, 13, 19144. DOI: 10.1038/s41598-023-46457-6.

Bruning, J.B., Murillo, A.C., Chacon, O., Barletta, R.G., Sacchettini, J.C., 2011. Structure of the *Mycobacterium tuberculosis* D-alanine: D-alanine ligase, a target of the antituberculosis drug D-cycloserine. Antimicrobial Agents and Chemotherapy, 55, 291–301. DOI: 10.1128/AAC.00558-10.

Chaachouay, N., Zidane, L., 2024. Plant-derived natural products: A source for drug discovery and development. Drugs and Drug Candidates, 3, 184–207. DOI: 10.3390/ddc3010011.

Chan, L.C., Gilbert, A., Basuino, L., da Costa, T.M., Hamilton, S.M., Dos Santos, K.R., Chambers, H.F., Chatterjee, S.S., 2016. PBP 4 mediates high-level resistance to new-generation cephalosporins in *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy, 60, 3934–3941. DOI: 10.1128/AAC.00358-16.

Contreras-Martel, C., Martins, A., Ecobichon, C., Trindade, D.M., Matteï, P.J., Hicham, S., Hardouin, P., Ghachi, M.E., Boneca, I.G., Dessen, A., 2017. Molecular architecture of the PBP2-MreC core bacterial cell wall synthesis complex. Nature Communications, 8, 776. DOI: 10.1038/s41467-017-00783-2.

Da Costa, T.M., De Oliveira, C.R., Chambers, H.F., Chatterjee, S.S.,018. PBP4: A new perspective on *Staphylococcus aureus* β-lactam resistance. Microorganisms, 6, 57. DOI: 10.3390/microorganisms6030057.

Galinier, A., Delan-Forino, C., Foulquier, E., Lakhal, H., Pompeo, F., 2023. Recent advances in peptidoglycan synthesis and regulation in bacteria. Biomolecules, 13, 720. DOI: 10.3390/biom13050720.

Gutbrod, P., Yang, W., Grujicic, G.V., Peisker, H., Gutbrod, K., Du, L.F., Dörmann, P., 2021. Phytol derived from chlorophyll hydrolysis in plants is metabolized via phytenal. Journal of Biological Chemistry, 296, 100530. DOI: 10.1016/j.jbc.2021.100530.

Gyawali, R., Bhattarai, B., Bajracharya, S., Bhandari, S., Bhetwal, P., Bogati, K., Neupane, S., Shrestha, S., Shrestha, A.K., Joshi, R., Paudel, P.N., 2020. α-amylase inhibition, antioxidant activity and phytochemical analysis of *Calotropis gigantea* (L.) Dryand. Journal of Health and Allied Sciences, 10, 77–81. DOI: 10.37107/jhas.143.

Harris, A.D., Samore, M.H., Lipsitch, M., Kaye, K.S., Perencevich, E., Carmeli, Y.,2002. Control-group selection importance in studies of antimicrobial resistance: Examples applied to Pseudomonas aeruginosa, Enterococci, and Escherichia coli. Clinical Infectious Diseases, 34, 1558–1563. DOI: 10.1086/340533, PubMed: 12032889.

Islam, M.T., Ali, E.S., Uddin, S.J., Shaw, S., Islam, M.A., Ahmed, M.I., Chandra Shill, M., Karmakar, U.K., Yarla, N.S., Khan, I.N., Billah, M.M., Pieczynska, M.D., Zengin, G., Malainer, C., Nicoletti, F., Gulei, D., Berindan-Neagoe, I., Apostolov, A., Banach, M., Yeung, A.W.K., El-Demerdash, A., Xiao, J., Dey, P., Yele, S., Jóźwik, A., Strzałkowska, N., Marchewka, J., Rengasamy, K.R.R., Horbańczuk, J., Kamal, M.A., Mubarak, M.S., Mishra, S.K., Shilpi, J.A., Atanasov, A.G., 2018. Phytol: A review of biomedical activities. Food and Chemical Toxicology, 121, 82–94. DOI: 10.1016/j.fct.2018.08.032.

Julius, O.O., Oluwasusi, V.O., Ibiyemi, M.F. and Oluwatobi, F.B., 2021. Antibacterial Activities and Phytochemical Screening of Crude Extracts of *Calotropis gigantea* (Giant Milk Weed). South Asian Journal of Research in Microbiology, 9(3), pp.24-31.

Kumar, H., Sharma, S., Vasudeva, N., 2021. Pharmacological profile of *Calotropis gigantea* in various diseases: A profound look. Int. J. Creat. Res Thoughts, 9, 2987–2996.

Lee, K., Kim, D., 2019. In-silico molecular binding prediction for human drug targets using deep neural multi-task learning. Genes, 10, 906. DOI: 10.3390/genes10110906, PubMed: 31703452.

Lima, S.L., Colombo, A.L., de Almeida Junior, J.N., 2019. Fungal cell wall: Emerging antifungals and drug resistance. Frontiers in Microbiology, 10, 2573. DOI: 10.3389/fmicb.2019.02573.

Loza-Mejía, M.A., Salazar, J.R., Sánchez-Tejeda, J.F., 2018. In silico studies on compounds derived from Calceolaria: Phenylethanoid glycosides as potential multitarget inhibitors for the development of pesticides. Biomolecules, 8, 121. DOI: 10.3390/biom8040121.

Masters, E.A., de Mesy Bentley, K.L., Gill, A.L., Hao, S.P., Galloway, C.A., Salminen, A.T., Guy, D.R., McGrath, J.L., Awad, H.A., Gill, S.R., Schwarz, E.M., 2020. Identification of penicillin binding protein 4 (PBP4) as a critical factor for *Staphylococcus aureus* bone invasion during osteomyelitis in mice. PLOS Pathogens, 16, e1008988. DOI: 10.1371/journal.ppat.1008988.

Mir, W.R., Bhat, B.A., Rather, M.A., Muzamil, S., Almilaibary, A., Alkhanani, M., Mir, M.A., 2022. Molecular docking analysis and evaluation of the antimicrobial properties of the constituents of *Geranium wallichianum* D. Don ex Sweet from Kashmir Himalaya. Scientific Reports, 12, 12547. DOI: 10.1038/s41598-022-16102-9.

Moon, T.M., D'Andréa, É.D., Lee, C.W., Soares, A., Jakoncic, J., Desbonnet, C., Garcia-Solache, M., Rice, L.B., Page, R., Peti, W., 2018. The structures of penicillin-binding protein 4 (PBP4) and PBP5 from *Enterococci* provide structural insights into β-lactam resistance. Journal of Biological Chemistry, 293, 18574–18584. DOI: 10.1074/jbc.RA118.006052.

Nasim, N., Sandeep, I.S., Mohanty, S., 2022. Plant-derived natural products for drug discovery: Current approaches and prospects. Nucleus (Calcutta), 65, 399–411. DOI: 10.1007/s13237-022-00405-3.

Nejhad A., Behbahani AB, Hojjati M, Vasiee A, Mehrnia MA., 2023. Identification of phytochemical, antioxidant, anticancer and antimicrobial potential of *Calotropis procera* leaf aqueous extract. Sci Rep 13, 14716.

Nkwocha, C., Felix, O.J., Idoko, N.R., 2023. GC-FID spectroscopic analysis and antioxidant activities of methanolic fraction of *Cassia tora* leaves. Pharmacological research. Modern Chinese Medicine, 9, 100338.

Nooshkam, M., Falah, F., Zareie, Z., Tabatabaei Yazdi, F., Shahidi, F., Mortazavi, S.A., 2019. Antioxidant potential and antimicrobial activity of chitosan–inulin conjugates obtained through the Maillard reaction. Food Science and Biotechnology, 28, 1861–1869. DOI: 10.1007/s10068-019-00635-3.

Onyeike, E.N., Egbuna, C., Patrick-Iwuanyanwu, K.C., 2023. Phytochemical screening and quantitative analysis of *Annona muricata* Leaf ethanolic extract by gas chromatography-flame ionization detection (GC-FID). IPS Journal of Drug Discovery Research and Reviews, 2, 1–4. DOI: 10.54117/ijddrr.v2i1.15.

Saddiq, A.A., Tag, H.M., Doleib, N.M., Salman, A.S., Hagagy, N., 2022. Antimicrobial, antigenotoxicity, and Characterization of *Calotropis procera* and Its Rhizosphere-Inhabiting Actinobacteria: In vitro and in vivo Studies. Molecules, 27, 3123. DOI: 10.3390/molecules27103123.

Salm, S., Rutz, J., van den Akker, M., Blaheta, R.A., Bachmeier, B.E., 2023. Current state of research on the clinical benefits of herbal medicines for non-life-threatening ailments. Frontiers in Pharmacology, 14, 1234701. DOI: 10.3389/fphar.2023.1234701.

Saxena, D., Maitra, R., Bormon, R., Czekanska, M., Meiers, J., Titz, A., Verma, S., Chopra, S., 2023. Tackling the outer membrane: Facilitating compound entry into Gram-negative bacterial pathogens. Npj Antimicrobials and Resistance, 1, 17. DOI: 10.1038/s44259-023-00016-1.

Shahidi, F., Tabatabaei Yazdi, F., Nooshkam, M., Zareie, Z., Fallah, F., 2020. Chemical modification of chitosan through non-enzymatic glycosylation reaction to improve its antimicrobial and anti-oxidative properties. Iran. Food Science and Technology Research J, 16, 117–129.

Sharma, A.D., Kaur, I., 2022a. A novel therapeutic eucalyptol from eucalyptus essential oil targeting penicillin binding proteins (PBPs) against gram-positive and gram-negative bacteria: Molecular docking and experimental approach. Arabian Journal of Medicinal and Aromatic Plants, 8, 24–42.

Sharma, A.D., Kaur, I., 2022b. Aroma profile and in-silico molecular docking studies of essential oil against "Aspergillosis" from *Eucalyptus polybrachtea*. South African Journal of Botany, 147: 754-763. https://doi.org/10.1016/j.sajb.2022.03.011

Singh, M., Javed, K., 2013. Chemical characterization and antimicrobial activity of *Calotropis gigantea* Linn. Flower essential oil collected from northern plain of India. International Journal of Advanced Biotechnology and Research, 4, 533–541.

Sripathi, R., Ravi, S., 2017. Molecular docking studies of the constituents present in the essential oil of *Plectranthus hadiensis* against bacterial proteins. International Journal of Chemical Sciences, 15, 185.

Straume, D., Piechowiak, K.W., Olsen, S., Stamsås, G.A., Berg, K.H., Kjos, M., Heggenhougen, M.V., Alcorlo, M., Hermoso, J.A., Håvarstein, L.S., 2020. Class A PBPs have a distinct and unique role in the construction of the pneumococcal cell wall. Proceedings of the National Academy of Sciences of the United States of America, 117, 6129–6138. DOI: 10.1073/pnas.1917820117.

Timilsina, H., Modi, B. and Basnyat, R., 2020. Phytochemical, antimicrobial and ethnobotanical study of *Calotropis gigantea.* Journal of Health and Allied Sciences, 10(2), pp.23-27.

Vaou, N., Stavropoulou, E., Voidarou, C.C., Tsakris, Z., Rozos, G., Tsigalou, C., Bezirtzoglou, E., 2022. Interactions between medical plant-derived bioactive compounds: Focus on antimicrobial combination effects. Antibiotics, 11, 1014. DOI: 10.3390/antibiotics11081014.

Wadhwani, B.D., Mali, D., Vyas, P., Nair, R., Khandelwal, P., 2021. A review on phytochemical constituents and pharmacological potential of *Calotropis procera*. RSC Adv. 4;11(57):35854-35878. doi: 10.1039/d1ra06703f

Wahba, H.E., Khalid, K.A., 2018. Comparative study on essential oil composition in various organs of Sodom apple (*Calotropis procera*) grown wild in Egypt. Asian Journal of Plant Sciences, 17, 85–90. DOI: 10.3923/ajps.2018.85.90.

Wijeweera, W.P.S.N., Senaratne, K.A.D.W., Dhileepan, K., 2021. Studies on the fruit feeding weevil, *Paramecops farinosa* (Coleoptera: Curculionidae. in Sri Lanka as a prospective weed biological control agent of invasive weed, *Calotropis* spp. Journal of Biological Control, 34, 241–250.

Yang, X., Zhang, X., Yang, S.P., Liu, W.Q., 2013. Evaluation of the antibacterial activity of patchouli oil. Iranian Journal of Pharmaceutical Research, 12, 307–316. PubMed: 24250637.

ABS

Original Research

Qualitative and Quantitative Detection of Virulence-Associated Genes in Escherichia Coli Isolates from Women with Urinary Tract Infections

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Abstract

Escherichia coli is the most common type of Enterobacteriaceae family that causes urinary tract infections. This bacterium causes diseases due to its many virulence factors. During the study, (150) urine samples were collected for women with symptoms of UTIs aged between (20-50) years from 1/9/2021 to 1/4/2022. Forty-five isolates of *E. coli* were diagnosed using a biochemical test, a VITEK2 system, and molecular detection using traditional PCR. A molecular study was conducted on 25 of these isolates using conventional PCR and qRT-PCR to investigate the presence of genes included (*16S rRNA*, *uidA*, *fimA* and *kpsMTII*). The traditional PCR results showed the presence of both *16S rRNA* and *uidA* gene in all 25 isolates of *E. coli*. In contrast, the results showed that 23 (92%) and 18 (72%) isolates possessed *fimA* and *kpsMTII* genes, respectively. Following the extraction of RNA from twenty-five pathogenic isolates of *E. coli* and ten non-pathogenic isolates, gene expression of *fimA* and *kpsMTII* was quantified using quantitative real-time reverse transcription PCR (qRT-PCR). The observed fold change indicates a potential overexpression of the *KSPMII* and *FimA* genes in uropathogenic *E. coli*. The expression of the *kpsMTII* gene showed the highest level of folding with an average of 22.89 in the case of uropathogenic *E.coli* isolates compared with non-pathogenic isolates (control) with an average of 4.11. While the expression of the *fimA* gene with the highest level of fold change with an average of 90.52 in the case of uropathogenic *E.coli* isolates compared with non-pathogenic isolates (control) with an average of 0.94, Significant differences were observed among the isolates, as indicated by P values less than 0.05. This finding demonstrated that qRT-PCR detects genes that were expressed in comparison with traditional PCR. Also, qRT-PCR is a more sensitive and accurate assay than conventional PCR for determining the quantitative prevalence of *E. coli* in urine samples.

Keywords

UPEC (uropathogenic *E. coli*); qRT-PCR; fimA, kpsMTII

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Uporaba klasičnega PCR in RT-PCR za odkrivanje nekaterih genov faktorjev virulence bakterij *Escherichia coli*, izoliranih iz pacientk z uroinfektom

Izvleček

Escherichia coli je najpogostejša vrsta iz družine Enterobacteriaceae, ki povzroča okužbo sečil. Ta bakterija lahko povzroči bolezni zaradi številnih dejavnikov virulence. Med študijo je bilo zbranih 150 vzorcev urina pri ženskah s simptomi infekcije sečil v starosti 20-50 let. Vzorci so bili nabrani v obdobju od 1.9.2021 do 1.4.2022. Diagnostično smo uporabili 45 izolatov *E. coli* z biokemičnim testom, sistemom VITEK2 in molekularno detekcijo s klasičnim PCR. Molekularna študija je bila izvedena na 25 od teh izolatov z uporabo klasičnega PCR in qRT-PCR za gene 16S rRNA, uidA, fimA in kpsMTII. Rezultati PCR so pokazali prisotnost 16S rRNA in gena uidA v vseh 25 izolatih *E. coli*, medtem ko so rezultati pokazali, da je imelo 23 (92 %), izolatov gen fimA in 18 (72 %) gen KSPMII. Po ekstrakciji RNA iz petindvajsetih patogenih izolatov *E. coli* in desetih nepatogenih izolatov smo ekspresijo genov kvantificirali s kvantitativno PCR v realnem času (qRT-PCR). Opazili smo potencialno čezmerno izražanje genov KSPMII in FimA v uropatogeni *E. coli*. Ekspresija gena KSPMII je pokazala najvišjo stopnjo ekspresije s povprečjem 22,89 v primeru uropatogenih izolatov *E.coli* v primerjavi z nepatogenimi izolati (kontrola) s povprečjem 4,113. Medtem ko smo najvišjo ekspresijo gena fimA s povprečjem 90,52 izmerili v primeru uropatogenih izolatov E.coli. Povprečje za nepatogene izolate (kontrola) je bilo 0,946. Naša raziskava je pokazala, da je qRT-PCR bolj občutljiv in natančen test kot klasični PCR za določanje kvantitativne prevalence *E. coli* v vzorcih urina.

Ključne besede

UPEC (uropatogena *E. coli*); qRT-PCR; fimA, kpsMTII

Introduction

The term urinary tract infections (UTIs) refer to the presence of bacteria which are not within the normal microbiota of the vagina, urethra and skin in the urinary tract environment with a significant number (≥105) cells/ml in the middle urine sample (Nicolle et al., 2019), these infections are a health problem in both the community and hospitals (Ahmed et al., 2019). In females, they are more common than males due to a variety of variables that occur in the female body, especially the urinary tract, such as shortness of the urethra and entry facilitated by pathogens through sexual contact, and the proximity of the anus to the vagina (Ahmed and Yousry, 2021), This area surrounding the rectum is hot and humid, which is teeming with living organisms, where bacteria can easily reach the bladder in females (Tille, 2014). The main culprit of UTIs is the Enterobacteriaceae family, the most common of which is *Escherichia coli*, which is responsible for about (90-80%) of all these infections worldwide (Zalmanovici et al., 2010; Dhahi, 2020). *E. coli* possesses several virulence factors that enable it to colonize and induce infection in the urinary tract, including the ability to adhere to urethral epithelial cells encoded by (sfa, fimA and pap). Alpha-hemolysin is a protein extracellular cytolytic toxin encoded by hlyA gene, whereas the kpsMTII gene encodes for capsule synthesis, the ompT gene encodes for protease in outer membranes, and has iron acquisition systems encoded by (fyuA, ironN, iucD and iutA). Virulence-associated genes (VAGs) are either carried on the chromosome or the plasmid (Dadi et al., 2020). Rapid and accurate diagnosis is a step towards treatment, and is done by using molecular techniques, including polymerase chain reaction (PCR) and quantitative real-time reverse transcription (qRT-PCR) techniques. The technique to screen bacteria, including *Escherichia coli*, relies on determine the possession of the gene encoding virulence factors and enhancing epidemiological information when investigating this bacterium (Maniam et al., 2022). This study aimed to use molecular detection by traditional PCR and qRT-PCR for analysing the presence of diagnostic genes included (16S rRNA and uidA) and some virulence factors genes (fimA, kpsMTII) of *E. coli* isolated from UTIs of women in Diyala/Iraq.

Materials and Methods

Bacterial Isolation

One hundred and fifty samples were taken from women with symptoms of UTIs their ages between (20-50) years from 1/9/2021 to 1/4/2022, Samples were collected from two hospitals in Diyala, Iraq, including Baquba General Hospital and Al-Batol Hospital for maternity and children. Forty-five isolates of E. coli were obtained. After cultured sample on multiple media, including Blood agar, MacConkey agar, and Chrom agar Orientation agar. The initial identification depended on conventional methods (biochemical test) and the VITEK2 system (bioMerieux, France). We used traditional PCR to detect the 16S rRNA and uidA genes for identification confirmation.

Detection of studied genes by traditional PCR

The instructions of the producing companies were applied for both the DNA extraction for isolates prepared from (Geneaid Thailand) and the preparation of the studied gene primers prepared from Macrogen (USA) for detection genes, including (16S rRNA, uidA, fimA and kpsMTII). The primers for the studied genes were clarified in Table 1. The mixture for the traditional PCR reaction for all genes consists of 1 µl forward and reverse primers for each gene at a concentration of 10 pmol/µl, two µl DNA, and 8.5 µl nuclease-free water to the microtubules containing the components (i-Taq) The final volume of the PCR reaction was 25 µl. The Thermal cycle was programmed individually for each gene, as shown in Table 2. Agarose gel was used for gene electrophoresis. After the migration expired, the stained bundles were visualized by a gel imaging system.

Extracted RNA from bacterial isolates was done according to the manufacturer's instructions using an extraction kit(Quick-RNA™, USA). Unique spin-column technology is used to produce high-quality total RNA (including small/microRNAs) prepared for RT/qPCR. EasyScript® First-Strand cDNA Synthesis SuperMix. To conduct the quantitative (qRT-PCR) by using Syber Green chemistry, the LunaScript qRT Master Mix Kit (5X) from BioLabs (England) was utilized. The reaction mixture was prepared with a total volume of 20 µl, consisting of 10 µl of the master mix, 0.5 µl each of the forward and reverse primers, 5 µl of cDNA from each sample, and 4 µl of nuclease-free water. The reaction was mixed thoroughly and centrifuged briefly to collect solutions at the bottom of the PCR tubes. Then, after the preparation, the tubes were placed into the thermal cycler programmed in Table 3. The qPCR protocol was initiated with polymerase activation at 95°C for 1 minute. This was followed by 45 amplification cycles, each consisting of a denaturation step at 95°C for 15 seconds and an annealing step at 60°C for 20 seconds, during which channel scanning was performed. Melting curve analysis was conducted based on the separation characteristics of double-stranded cDNA during cycles with progressively increasing denaturation temperatures. The Ct values of the target genes (kpsMTII and fimA) were normalized to the 16S RNA reference gene, and The expression levels of the target genes were quantified using the relative quantification approach, utilizing the comparative Ct method combined with fold change analysis, precisely calculated as 2^-ΔΔCt.

Table 1. The primers of the studied genes.

Tabela 1. Primeri preučevanih genov.

Primers Name	Primer Sequence	Product size (bp)	Reference
16S rRNA	F: AGA GTT GAT CCT GGC TCA	1500	(Maleki et al., 2017)
	R: GGT TAC CTT GTT ACG ACTT		
uidA	F: CAT TAC GGC AAA GTG TGG GTC AAT	658	(Adamus-Bialek et al., 2009)
	R: CCA TCA GCA CGT TAT CGA ATC CTT		
fimA	F: GTT GTT CTG TCG GCT CTG TC	447	(Hasan et al., 2021)
	R: ATG GTG TTG GTT CCG TTA TTC		
kpsMTII	F: GCG CAT TTG CTG ATA CTG TTG	292	(Johnson and Stell, 2000)
	R: CAT CCA GAC GAT AAG CAT GAG CA		

Table 2. PCR program for studied genes.

Tabela 2. Program PCR za preučevane gene.

Genes	Denaturation	Annealing	Extension*	No. Cycles
16S rRNA	94°C / 30 sec	55 °C / 30 sec	72 °C/30 sec	35
uidA	92°C/60 sec	58 °C / 60 sec	72 °C/30 sec	35
fimA	94°C/45 sec	55 °C / 45 sec	72 °C/54 sec	30
KPSMTII	94°C/30 sec	60°C/ 60 sec	72°C/60 sec	30

*Initial Denaturation 94°C\ 5 min and final extension 72°C\ 10 min for all genes. **

Table 3. qRT-PCR program.

Tabela 3. Program qRT-PCR

qPCR Steps	Temp.	Time	Cycle(s)
Initial activation	95°C	10min	1
Denaturation	95°C	15 sec	45
Annealing	60°C	20 sec	
Extension	90°C	15 sec	

Results

The results of this study obtained 45(30%) *E. coli* isolates from women suffering from UTIs using a conventional VITEK2 system and molecular methods for identification After obtaining ethical approval to take urine samples from patients. A molecular study was conducted on 25 of these isolates using traditional PCR and qRT-PCR to investigate the presence of genes included (16S rRNA, uidA, fimA and kpsMTII). The conventional PCR results showed the presence of (16S rRNA and uidA) which diagnostic gene in all 25 isolates of *E. coli*. In contrast, 23 (92%) and 18 (72%) isolates possessed fimA and kpsMTII genes, respectively. Molecular detection results for all genes using traditional PCR are presented in Table 4. A significant statistical difference ($p < 0.05$) between isolates carrying each gene and isolates was observed for fimA and kpsMTII.

Real-time qRT-PCR quantification of kpsMTII and fimA gene expression

The quantitative qRT-PCR reaction experiment used (25) pathogenic and (10) non-pathogenic isolates. Melting curve analysis was carried out based on the separation properties of double-stranded cDNA during cycles with progressively increasing denaturation temperatures (Tm). The assay was conducted in triplicate for each sample, and the average of these three measurements was used to quantify the gene expression for that sample. Ct value of target genes (kpsMTII and fimA) had been standardized to 16sRNA reference genes, and the relative quantitative method was used to determine the target. To represent gene expression using the comparative Ct formula and Fold change analysis (2^-ΔΔCT), you can create a table with the following figure (5).

Expression of fimA and kpsMTII in the pathogenic *E. coli* was significantly higher than in non-pathogenic strains (Fig. 1).

The expression of the kpsMTII gene exhibited the highest level of upregulation, with a mean value of 22.8943, in uropathogenic *E. coli* isolates compared to non-pathogenic isolates (control), with a mean value of 4.113. Statistical analysis revealed significant differences between the groups, with P-values < 0.05.

Figure 1 shows the expression of the fimA gene with the highest level of fold change with an average of 90.52 in the case of uropathogenic *E. coli* isolates compared with non-pathogenic isolates (control) with an average of 0.946. Significant differences between the isolates were observed, with P values < 0.05.

Table 4. Gene prevalence used traditional PCR.

Tabela 4. Razširjenost genov, uporabljenih pri tradicionalni PCR.

	16SrRNA	uidA	fimA	kpsMTII
No. isolates	25	25	25	25
Carry gene (+)	25(100%)	25(100%)	23(92%)	18(72%)
Not carry gene	0 (0%)	0 (0%)	2 (8%)	7(28%)
P value	1.00	1.00	P<0.001***	P<0.001***

Table 5. The gene expression of kpsMTII AND fimA in Pathogenic and non-pathogenic *E.coli.*

Tabela 5. Izražanje genov kpsMTII IN fimA v patogenih in nepatogenih *E.coli*.

Groups	N	ΔCT (Mean±SE)	ΔΔCT (Mean±SE)	Fold Change (2^- ΔΔCT) Mean±SE
Control kpsMTII	10	7.01±0.76	5.00± 0.31	4.11±0.45
Control fimA	10	18.21±1.46	11.36±1.04	0.94±0.11
kpsMTII	25	6.67±0.94	-0.42±0.12*	22.89±1.62**
fimA	25	14.23±1.04	-4.44±0.34	90.52±3.46**

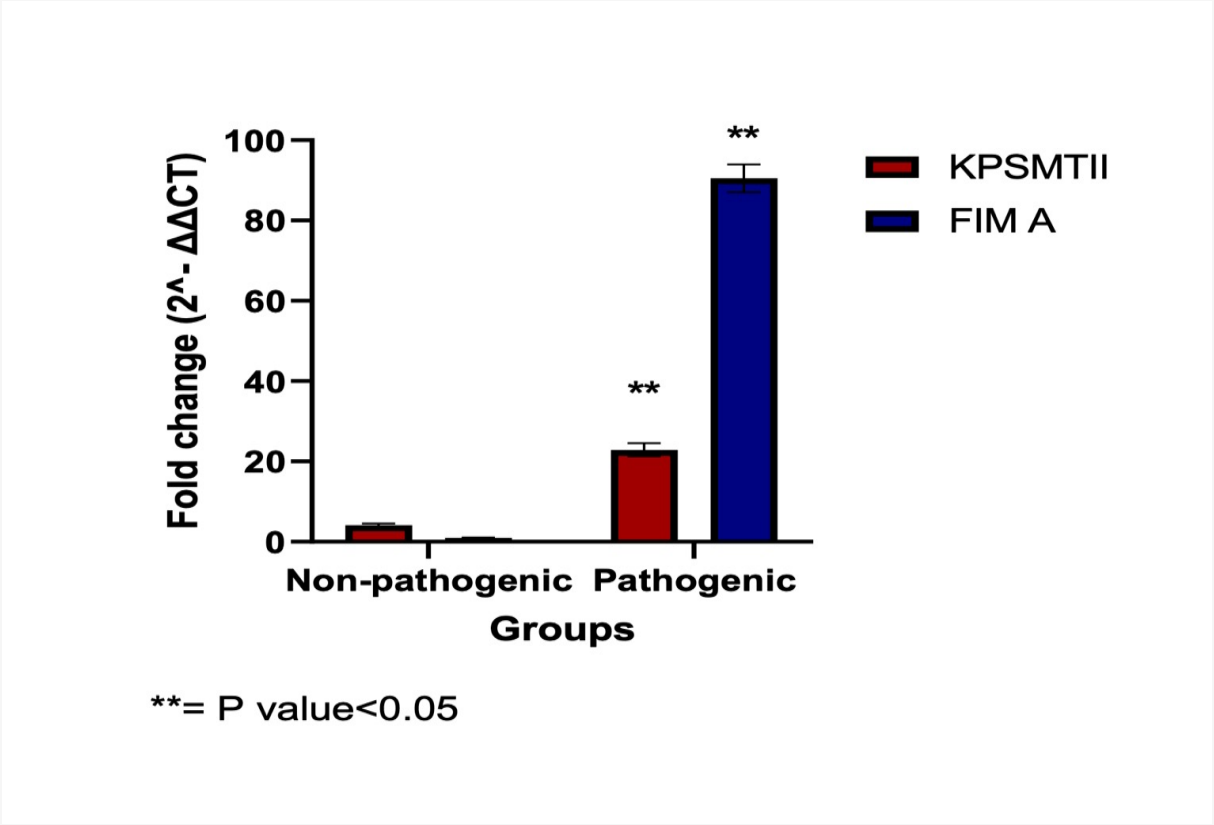

Figure 1. Folding change of kpsMTII and fimA gene expression.

Slika 1. Spremembe prepogibanja kpsMTII in izražanje gena fimA.

Discussion

Urinary tract infections are the most common bacterial infection, affecting all age groups, especially women, *E. coli* is the most common cause, followed by *Klebsiella* (Abalkhail et al., 2022). The results obtained in this study are consistent with the results of other studies, including Thanoun (2022) from Mosul, Ahmed et al. (2019) from Saudi Arabia, and Mathai et al. (2001) from America, if the percentage of infection with this bacteria was 33.1%, 27%, 46.9%, respectively.

Molecular results for 16SrRNA and uidA genes using traditional PCR explained that all isolates possess them, which also agrees with the researcher Salih (2015). Therefore, it can be considered an effective diagnostic method for identifying the presence of *E. coli* in patients with urinary tract infections, confirmed through isolation and subsequent analysis. This was similared withe Forbes et al. (2007) and Brooks et al. (2010) that the final diagnosis of *E. coli* was made based on molecular methods, especially the 16SrRNA gene. The uidA gene is a frequently used enzyme marker for detecting these bacteria in newly developed detection media (Brons et al., 2020) as this gene encodes an enzyme specific to *E. coli*, the enzyme beta-D-glucuronidase (Cleuziat and Robert-Baudouy, 1990). This gene is used to diagnose UPEC, and the results of this study were confirmed to those of Brons et al. (2020), in which all isolates possessed uidA gene.

All twenty-five pathogenic isolates expressed target genes (kpsMTII and fimA) by using qRT-PCR in different levels of fold change, While by using traditional PCR, the results showed that 23(92%), 18(72%) of isolates possessed fimA and kpsMTII genes respectively. This result demonstrated that the qRT-PCR technique was Quantitative PCR (qPCR) demonstrates superior sensitivity and specificity compared to conventional PCR for assessing the presence of *E. coli* in UTI specimens. The fimA adhesion virulence gene in these isolates is implicated in enhancing the colonization and persistence of *E. coli* in the urinary tract. Additionally, the expression of kpsMTII genes, which are crucial for capsule synthesis, was significantly elevated in these isolates. These genes have been associated with increased pathogenicity, a higher probability of antibiotic resistance, and biofilm formation. Implementing qRT-PCR for some genes in clinical practice may facilitate the identification of individuals at elevated risk for (UTIs) with UPEC and contribute to reducing antibiotic usage.

The creation of UTI in the host is made more accessible by the UPEC's capacity to acquire the genes that code for virulence, allowing it to persist continue in the urinary system. Consequently, the development and expression of the genes that code for virulence are frequently linked to the severity of clinical UTI symptoms (Stork et al., 2018). Identifying UPEC-associated virulence genes in *E. coli* strains can inform the decision-making process regarding the necessity of antibiotic treatment and predict the likelihood of asymptomatic bacteriuria progressing to symptomatic urinary tract infections (UTIs). This is crucial as evidence suggests that bacterial colonization may play a role in preventing symptomatic UTIs (Salvador et al., 2012; Maniam et al., 2022). All these factors emphasis the importance need to identify the specific characteristics of the organism, including its virulence traits, before initiating therapy with *E. coli* strains.

qRT-PCR is a highly sensitive and powerful method for analyzing gene expression levels of genes. Comprehending its technical components is necessary to utilise its quantitative capabilities fully. Quantitative qRT-PCR necessitates the correction of experimental differences in individual qRT and PCR efficiency for successful results (Pakbin et al., 2023).

Conclusions

Quantitative Real-Time PCR (qRT-PCR) utilizing SYBR Green melting curve analysis has proven to be a highly sensitive and specific method with a low detection threshold for identifying and quantifying *E. coli* isolates in urine samples.

Author Contributions

Conceptualization, Z.A.H; methodology, Z.A.H.; software, L.A.S.A.; validation, Z.H.M.; investigation, S.A.J.; writin – original draft preparation, Z.H.M.; writing – review and editing, L.A.S.A.; visualization, S.A.J.; supervision, Z.H.M. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

References

Abalkhail, A., AlYami, A. S., Alrashedi, S. F., Almushayqih, K. M., Alslamah, T., Alsalamah, Y. A., Elbehiry, A., 2022. The prevalence of multidrug-resistant *Escherichia coli* producing ESBL among male and female patients with urinary tract infections in Riyadh Region, Saudi Arabia. InHealthcare 10(9), 1778.

Adamus-Bialek, W., Wojtasik, A., Majchrzak, M., Sosnowski, M., Parniewski, P., 2009. (CGG)4-based PCR as a novel tool for discrimination of uropathogenic *Escherichia coli* strains: comparison with enterobacterial repetitive intergenic consensus-PCR. J Clin Microbiol, (12), 3937-44.

Ahmed, M.G., Yosry, L.M., 2021. Genotypic Detection of the Virulence Factors of Uropathogenic *Escherichia coli* (UPEC) Strains Isolated from Pregnant Females and Their Correlation with Antibiotic Resistance Pattern. Al-Azhar Journal of Pharmaceutical Sciences, 63, 149 – 165.

Ahmed, N.A., Mahmood, S.S., Abbas, A.H., 2019. A Comparative Study Of Some Virulence Factors And Phylogenetic Charactrization Of Escherichia coli Isolates Causing Urinary Tract Infection And The Commensal Gut Microbiota. Iraqi Journal of Agricultural Sciences, 50(4), 1193–1198.

Brons. J.K., Vink. S.N., de- Vos, M.G.J., Reuter, S., Dobrindt. U., van- Elsas J.D., 2020. Fast identification of *Escherichia coli* in urinary tract infections using a virulence gene based PCR approach in a novel thermal cycler. J Microbiol Methods, 169, 105799.

Brooks, G.F., Carroll, K.C., Butel, J.S., Morse, S.A., Mietzner, T.A., 2010. Medical Microbiology. 25th ed. United States.

Cleuziat, P., Robert-Baudouy, J., 1990. Specific detection of *Escherichia coli* and *Shigella* species using fragments of genes coding for beta-glucuronidase. FEMS Microbiol Lett, 60(3):315-22.

Dadi, B.R., Abebe, T., Zhang, L., Mihret, A., Abebe, W., Amogne, W., 2020. Distribution of virulence genes and phylogenetics of uropathogenic *Escherichia coli* among urinary tract infection patients in Addis Ababa, Ethiopia. BMC Infectious Diseases, 20(1), 1–12. DOI: 10.1186/s12879-020-4844-z.

Dhahi, M.A.R., 2020. Sequencing Analysis of Pyelonephritis-Associated Pili Gene of Uro-Pathogenic *Escherichia coli* Isolated from Iraqi Patients-Baghdad. Biomed Pharma J., 13(2), 1011-1019.

Forbes, B.A., Sahm, D.F., Weissfeld, A.S., 2007. Bailey and Scott's Diagnostic Microbiology. International Edition. 12th ed. Mosby Elsevier.Houston, Texas.

Hasan, A.R., Al-Jubori, S. and Salman, A.S., 2021. Molecular Analysis of fimA Operon Genes among UPEC Local Isolates in Baghdad City. Archives of Razi Institute, 76(4), 829-840.

Johnson, J.R., Stell, A.L., 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J Infect Dis,181(1), 261-72.

Maleki, D., Jahromy, S. H., Karizi, S.Z. and Eslami, P., 2017. The Prevalence of acrA and acrB Genes Among Multiple-Drug Resistant Uropathogenic *Escherichia coli* Isolated from Patients with UTI in Milad Hospital, Tehran. Avicenna J Clin Microb Infec. 4(1), 1-7.

Maniam, L., Vellasamy, K.M., Jindal, H.M., Narayanan, V., Danaee, M., Vadivelu, J., 2022. Demonstrating the utility of *Escherichia coli* asymptomatic bacteriuria isolates' virulence profile towards diagnosis and management—A preliminary analysis. PLoS ONE 17(5): e0267296.

Mathai, D., Jones, R.N., Pfaller, M.A., 2001. Epidemiology and frequency of resistance among pathogens causing urinary tract infection in 1,510 hospitalized patients: a report from the sentry antimicrobial surveillance program (North America) Diagn Microbiol Infect Dis J, 40(3): 129-136 .

Nicolle, L.E, Gupta, K. Bradley, S.F. Colgan, R. DeMuri, G.P, Drekonja D, Eckert, L.O, Geerlings, S.E, Köves, B, Hooton, T.M., Juthani-Mehta, M., 2019. Clinical practice guideline for the management of asymptomatic bacteriuria: 2019 update by the Infectious Diseases Society of America. Clinical Infectious Diseases. 2,68(10): e83-110.

Pakbin, B., Brück, W. M., Brück, T. B., Allahyari, S., Ashrafi Tamai, I., 2023. A quantitative prevalence of Escherichia coli O157 in different food samples using real-time qPCR method. Food Science & Nutrition, 11(1), 228-235.

Tille, P.M. (2014). Bailey and Scott's Diagnostic microbiology. 13th ed. Mosby Elsevier. China. pp .1193.

Salih, E., Nader, M., Rasheed, M., 2015. Rapid Detection of Uropathogenic *Escherichia coli*. World Journal of Pharmaceutical Research, 4(12), 507–515.

Salvador, E., Wagenlehner, F., Köhler, C. D., Mellmann, A., Hacker, J., Svanborg, C., Dobrindt, U., 2012. Comparison of asymptomatic bacteriuria *Escherichia coli* isolates from healthy individuals versus those from hospital patients shows that long-term bladder colonization selects for attenuated virulence phenotypes. Infection and immunity, 80(2), 668-678.

Stork, C., Kovács, B., Rózsai, B., Putze, J., Kiel, M., Dorn, Á., and Dobrindt, U., 2018. Characterization of asymptomatic bacteriuria Escherichia coli isolates in search of alternative strains for efficient bacterial interference against uropathogens. Frontiers in microbiology, 9, 214.

Thanoun, Z.H.D., 2022. Isolation and identification of bacterial species that cause urinary tract infections in women, study of their virulence factors and molecular diagnosis. Master Thesis, College of Education for Pure Sciences, University of Mosul.

Zalmanovici, T.A., Green, H., Paul, M., Yaphe, J., Leibovici, L., 2010. Antimicrobial agents for treating uncomplicated urinary tract infection in women. Cochrane Database Syst. Rev,10:CD007182.

ABS

Original Research

Impact of water deficit on the anatomical structure of more productive and less productive cashew trees (*Anacardium occidentale* L.) in Côte d'Ivoire

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Abstract

This study aimed to understand the mechanism of resilience of the anatomical tissues of organs of the cashew tree in water deficit in the different climatic zones of Côte d'Ivoire. To achieve this, samples of leaves and roots were taken from 55 cashew trees considered high producers (APHP) and 164 neighbouring less productive trees (non-APHP) during the dry and rainy seasons. The technique of double staining with green-carmino made it possible to obtain anatomical sections of transversely cut samples under an optical microscope. The thicknesses and surface areas of the sections were measured using Image J software. The thickness of the leaf parenchymas of APHP trees did not show any variation according to the season, while the stomatal density increased during the dry season. The thickness of the leaves cortical and medullary parenchymas of non-APHP trees did not show any variation according to the season too, but the non-APHPs' palisade parenchyma thickness increased while their lacunar parenchyma thickness decreased from 37 ± 1.5 µm to 36 ± 3.6 µm during the dry season. The thickness of leaves on both sides of the epidermis increased while the thickness of xylem bundles decreased during the dry season in both APHP and non-APHP trees. The number of the roots' xylem vessels of APHP trees increased from 11 to 27 vessels/mm^2, while those of non-APHP trees increased from 15 to 41 vessels/mm^2 during the rainy season. The surface area of the midrib medullary parenchyma of APHP trees increased from 4300 ± 837 µm^2 to 5800 ± 412 µm^2 while those of non-APHP trees increased from 3400 ± 809 µm^2 to 5200 ± 993 µm^2 during the dry season. The thicknesses of the tissues of APHPs remained greater than those of non-APHPs, regardless of the sampling season.

Keywords

Cashew tree, anatomy, adaptation, water deficit, Côte d'Ivoire

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Vpliv pomanjkanja vode na anatomsko strukturo bolj produktivnih in manj produktivnih dreves indijskega oreščka (*Anacardium occidentale* L.) v Slonokoščeni obali

Izvleček

Namen naše raziskave je bil razumeti mehanizem odpornosti anatomskih tkiv organov drevesa indijskega oreščka na pomanjkanje vode v različnih podnebnih območjih Slonokoščene obale. Za dosego tega cilja so bili med suho in deževno sezono odvzeti vzorci listov in korenin pri 55 drevesih indijskega oreščka, ki veljajo za visoko produktivna (APHP), ter pri 164 sosednjih manj produktivnih drevesih. Tehnika dvojnega barvanja z zelenim karminom je omogočila pridobitev anatomskih prerezov prečno rezanih vzorcev, ki smo jih opazovali pod optičnim mikroskopom. Debeline in površine prerezov so bile izmerjene z uporabo programske opreme Image J. Debelina parenhima listov pri drevesih APHP ni kazala sezonskih sprememb, medtem ko se je gostota rež povečala v sušni sezoni. Prav tako se debelina kortikalnega in medularnega parenhima listov dreves brez APHP ni spreminjala glede na sezono, vendar se je debelina njihovega palisadnega parenhima povečala, medtem ko se je debelina lakunarnega parenhima zmanjšala s 37 ± 1,5 µm na 36 ± 3,6 µm v sušni sezoni. Debelina epidermisa na obeh straneh listov se je povečala, medtem ko se je debelina ksilema zmanjšala v sušni sezoni pri drevesih APHP in ne-APHP. Število ksilemskih elementov v koreninah dreves APHP se je povečalo z 11 na 27 žil/mm^2, pri drevesih brez APHP pa s 15 na 41 žil/mm^2 v deževni sezoni. Površina medularnega parenhima glavne žile korenin pri drevesih APHP se je povečala s 4300 ± 837 µm^2 na 5800 ± 412 µm^2, medtem ko se je pri drevesih brez APHP povečala s 3400 ± 809 µm^2 na 5200 ± 993 µm^2 v sušni sezoni. Debeline tkiv pri drevesih APHP so ostale večje kot pri drevesih brez APHP, ne glede na sezono vzorčenja.

Ključne besede

Indijski orešček, anatomija, prilagoditev, pomanjkanje vode, Slonokoščena obala

Introduction

The cashew tree (*Anacardium occidentale* L.) belongs to the clades Angiospermae and Eudicotidae, the order Sapindales and the Anacardiaceae family (APG 2016). Cashew cultivation constitutes an important new source of income for African farmers, who account for more than 55% of the global cashew nut production. Côte d'Ivoire is the leading African producer and exporter of raw cashew nuts (Henry et al. 2011) and the world's third biggest producer and exporter of raw cashew nuts after India and Vietnam (FAO 2024). Its production of raw cashew nuts was estimated at 970,000 tons in 2022 (FAO 2024). Despite this performance and constant evolution, Ivorian orchard yields are between 350 to 500 kg/ha (Djaha et al. 2010, FAO 2024) compared to those obtained in India, Vietnam, Brazil and Tanzania, which are between 1,000 and 1,500 kg/ha (Kiwuso et al. 2013). Cashew nut producers encounter enormous difficulties due to several factors, such as insufficient technical supervision, the use of low-yielding varieties and unknown sources (Charahabil et al. 2017).

In Côte d'Ivoire, the mass selection technique was used to identify Potentially High-Producing APHP Cashew Trees (Konan 2016). These trees were selected on the basis of the following characteristics: good tree architecture, precocity and grouped maturity, shape and quality of nuts, nut weight ≥ 7 g and yield ≥ 10 kg of nuts/tree/year. However, some studies have pointed out the existence of a relationship between the anatomy and the production potential of fruit trees. However, abiotic factors are the trigger for stress. Plant responses to these stresses are either plastic and reversible or irreversible (Skirycz and Inze 2010, Cramer et al. 2011). Indeed, the leaf is the most adaptable organ in its response to environmental conditions (Marchi et al. 2008). Leaf structures reflect the effects of water stress more clearly than the structures of stems and/or roots, whose effects are also visible.

This work aimed to understand the resilience mecha-

nisms of leaf and root tissues of Potentially High-Producing Cashew Trees (APHP) and their neighbours (non-APHP) in the cashew basin of Côte d'Ivoire according to different seasons.

Material and methods

Study areas

This study was carried out in the cashew basin of Côte d'Ivoire across 15 administrative regions (Figure 1) and two agro-ecological zones, characterized as follows:

- Guinean Sector, which is a transition zone between the forest zone in the south and the grassy savannah of the centre, with a dry season from January to February, a major rainy season from March to October and a small rainy season from November to December (FAO 2005, Konaté 2021). The annual rainfall is between 1,031.5 mm and 1,189.36 mm, while the temperature varies between 16 °C and 36 °C, with an average of 27 °C (FAO 2005, Ouattara et al. 2016);
- The Sudanian Sector is characterized by the north savannah vegetation, with a dry season from November to February and a long rainy season from March to October (FAO 2005, Konaté 2021). The annual rainfall is between 1,316.19 mm and 1,708.39 mm, and the temperature varies between 28 °C and 32 °C (FAO 2005, Ouattara et al. 2016).

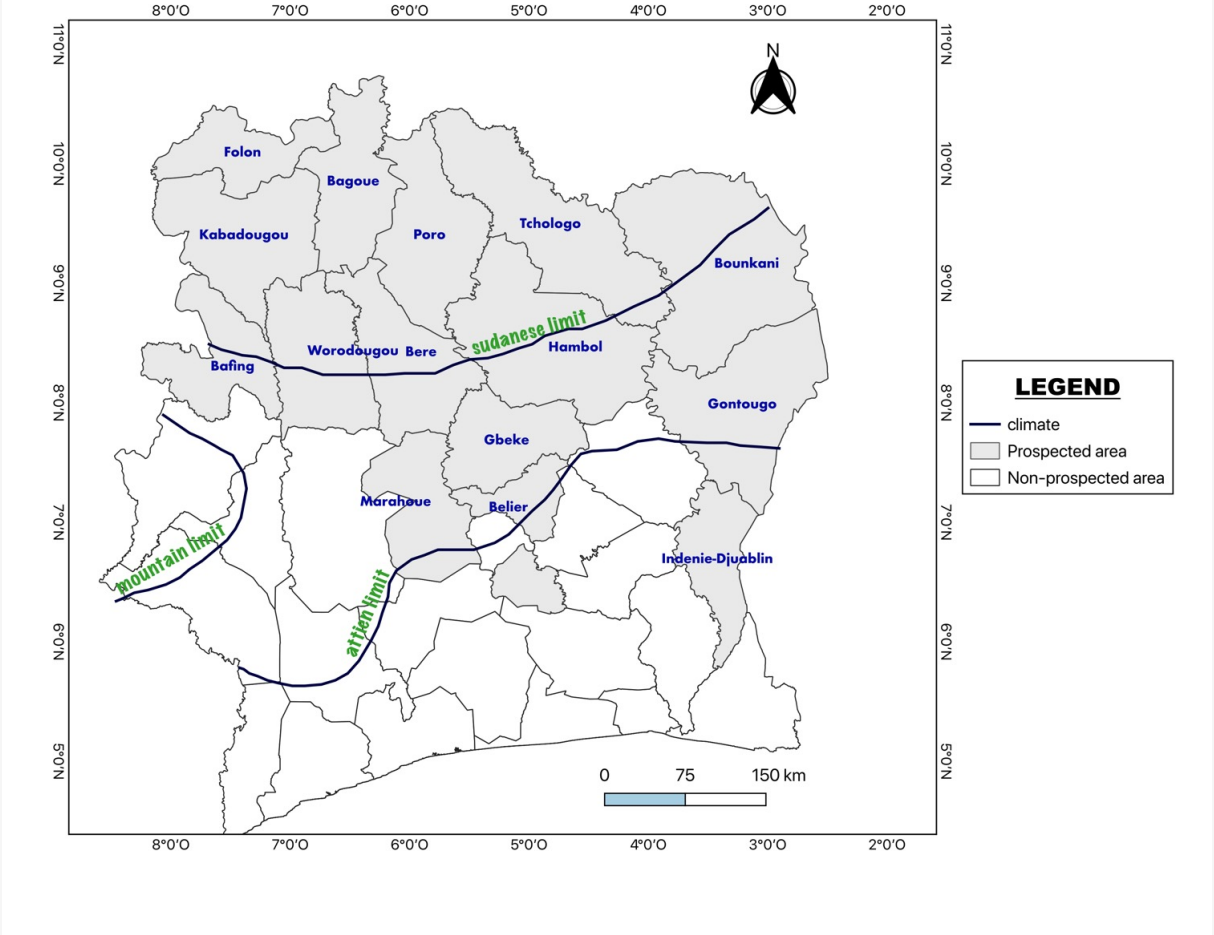

Figure 1. Map of the sample collection area.

Slika 1. Zemljevid območja zbiranja vzorcev.

Data collection

The samples of mature leaves and roots were collected according to the method of Sankharé (2018) from 219 cashew trees, including 55 that are known to be potentially more productive (APHP) and their 164 less productive immediate neighbours (non-APHP) in the four cardinal directions, over 15 administrative regions of the cashew production basin in Côte d'Ivoire (Figure 1). The leaves about 10-12 cm long and 6-8 cm wide, and the roots with a diameter of about 0.2-0.4 mm were collected during the dry season (February-March) and during the rainy season (July- August). These samples were stored in 70% alcohol before and during their preparation and observation under an optical microscope. Using a manual microtome and polystyrene, 0.1-0.2 mm microscopic sections of leaves, blade veins, midlines and roots were cut and stained using the classic green-carmino double staining technique (Ruzin 1999). To observe the stomata, a razor blade was placed on the lower surfaces of the leaves, and then the transparent epidermis located on the surfaces of the leaves was gently lifted. The fragments of the obtained transparent epidermis constituted the samples that were observed (Djinet et al. 2016). Anatomical tissues were visualized and recorded using Leica Acquire software.

Image analysis

The captured images were saved in 640×480 pixels format. Each anatomical section was measured with a micrometre graduated in millimetres (mm) and then converted to micrometres (µm) before observation under a microscope. Afterwards, the image of each anatomical section was measured in pixels at 10×, 20×, 40× and 100× magnifications. Therefore, calibration was done on each of the sections using this process to convert the photographs of the sections into µm.

Histological analysis

The anatomical tissues of leaves and roots were analyzed and measured with the Image-J 1.53t software. The parameters taken into account were:

- The thickness of the parenchymas, where the absorbed water is used for the photosynthetic activities;
- the structure of the epidermis that regulates water exchange between the interior and the exterior of the cashew aerial organs;
- the stomatal density and the surface of the opening of the ostiole of the stomata, which allow gas and water exchange between the interior and the exterior of the aerial organs of the cashew tree:

$$DS = \frac{Number\ of\ stomata}{\text{Leaf surface}}$$

- The characteristics of the xylems that conduct the absorbed water toward the cashew organs. Regarding the leaf midrib xylem diameter, the diagonal diameters were measured as recommended (Stein et al. 2017). The diameters of the root xylem vessels were measured taking into account both the tangential diameter (DT) and the radial diameter (DR); their ratio (R) was calculated by the following expression:

$$R = \frac{DT}{DR}$$

- The number of root xylem vessels was determined with the cell counter tool on 1 area of 1×1 mm (Grishaguin 1985).

Statistical analysis

The data collected in the Excel spreadsheet were analyzed using the R software to compare thicknesses and visualize them. The ANOVA test and Student t-test were performed to compare the data during the rainy and the dry seasons at the 5% significance level ($p < 0.05$). Pearson's correlation test was performed to assess the relation between the number of xylem vessels and the ratio of the tangential and radial diameters of the vessels of the roots.

Results

Leaf tissues

Parenchymas

The average thicknesses of the lacunar parenchyma (Figure 2) of both APHPs and non-APHPs were larger during the rainy season than during the dry season (Table 1, Figure 3). The season did not show any impact ($P = 0.090$) on the thickness of the APHPs' lacunar parenchyma, while drought showed a high negative impact ($P = 0.009$) on the thickness of the non-APHPs' lacunar parenchyma, which decreased from 37 ± 1.5 µm during the rainy season to 36 ±

3.6 µm during the dry season (Table 1).

The season did not show any impact (P > 0.105) on the thicknesses of the APHPs' lower and upper palisade parenchyma, while drought showed a high positive impact (P < 0.028) on the thicknesses of the non-APHPs' palisade parenchymas (Table 1) which increased from 54 ± 4.4 µm during the rainy season to 59 ± 5.4 µm during the dry season at the upper face, and from 21 ± 10.9 µm during the rainy season to 22 ± 5.2 µm during the dry season at the lower face (Table 1, Figure 3).

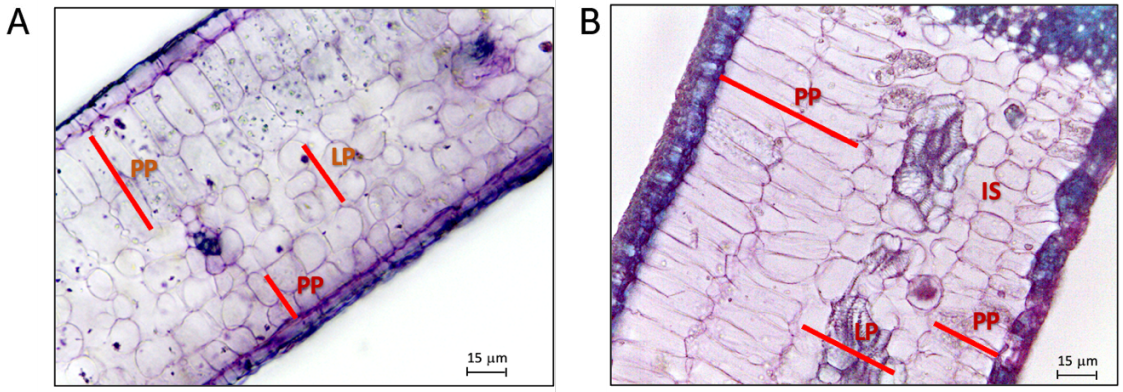

Figure 2. Pictures of the flattened blade of the cashew leaves according to the seasons (100× magnification). A: dry season; B: rainy season; PP: palisade parenchyma; LP: lacunar parenchyma; IS: intercellular space

Slika 2. Slike sploščene listne ploskve indijskega oreščka glede na letni čas (100-kratna povečava). A: suha sezona; B: deževna sezona; PP: palisadni parenhim; LP: lakunarni parenhim; IS: medcelični prostor

Table 1. Average thickness of leaf tissues according to the cashew tree categories and to the seasons. APHP: potentially higher-producing cashew trees. For the tissue, paired data with the same letter (P-value > 5%) are statistically similar, while data with different letters are different (5% > P-value > 1%) or highly different (1% > P-value > 0.1%).

Tabela 1. Povprečna debelina listnih tkiv glede na kategorije indijskih dreves in letne čase. APHP: drevesa indijskega oreščka s potencialno višjo rodnostjo. Pri tkivu so si parni podatki z isto črko (P-vrednost > 5 %) statistično podobni, medtem ko so podatki z različnimi črkami različni (5 % > P-vrednost > 1 %) ali zelo različni (1 % > P-vrednost > 0,1 %).

Tissue location		Flattened blade tissues					Midrib tissues			
Tree categories	Season type	Upper palisade parenchyma (µm)	Lower palisade parenchyma (µm)	Lacunar parenchyma (µm)	Upper epidermis (µm)	Lower epidermis (µm)	Upper cortical parenchyma (µm)	Lower cortical parenchyma (µm)	Medullary parenchyma (µm²)	Xylem bundles (µm)
APHP	Rainy season	52 ± 4.2[a]	20 ± 2.9[a]	40 ± 4.0[a]	5.6 ± 0.4[a]	5 ± 0.4[a]	8 ± 2.3[a]	18.5 ± 4.5[a]	4300 ± 837[a]	6.5± 0.7[a]
	Dry season	52 ± 6.3[a]	28 ± 2.1[a]	30± 6.3[a]	6.2 ± 1.0[b]	5.6 ± 1.1[b]	6.3 ± 0.8[a]	22 ± 3.4[a]	5800± 412[a]	6.4± 0.5[b]
	P-value	**0.500**	**0.105**	**0.090**	**0.032**	**0.036**	**0.075**	**0.055**	**0.094**	**0.005**
Non-APHP	Rainy season	54 ± 4.4[a]	21 ± 10.9[a]	37 ± 1.5[a]	6.4 ± 0.3[a]	5 ± 0.4[a]	7.2 ± 1.2[a]	15 ± 4.9[a]	3400 ± 809[a]	6.7 ± 0.9[a]
	Dry season	59 ± 5.4[b]	22± 5.2[b]	36± 3.6[b]	6.5 ± 1.1[b]	5.5 ± 0.7[b]	6.2± 0.5[b]	20 ± 2.0[a]	5200 ± 993[a]	5.9 ± 0.6[b]
	P-value	**0.028**	**0.015**	**0.009**	**0.005**	**0.030**	**0.047**	**0.090**	**0.131**	**0.040**

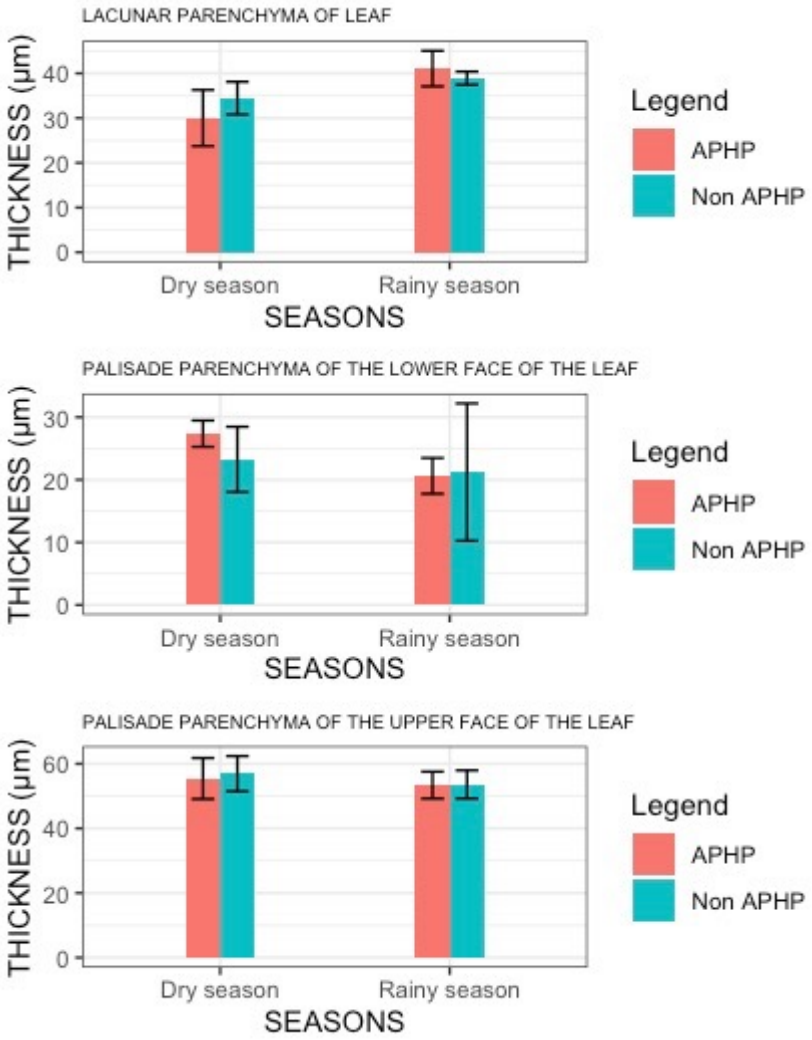

Figure 3. Thickness of the parenchyma of flattened leaf blades. IE: lower palisade parenchyma; SE: upper palisade parenchyma.

Slika 3. Debelina parenhima sploščenih listnih lopatic. IE: spodnji palisadni parenhim; SE: zgornji palisadni parenhim.

No impact ($P > 0.055$) of the season was found on the thicknesses of the APHPs' midrib in both face cortical parenchymas and on the non-APHPs' lower face cortical parenchyma thickness ($P = 0.090$), while drought showed a negative impact ($P = 0.047$) on the thickness of the non-APHPs' upper face cortical parenchyma which decreased from 7.2 ± 1.2 µm during the rainy season to 6.2 ± 0.5 µm during the dry season (Table 1, Figure 4).

The season did not show any impact ($P > 0.093$) on the area of the medullary parenchyma of both APHPs and non-APHPs, even if the average area of the medullary parenchyma of the APHPs was about 4,300 ± 837 µm^2 during the rainy season and 5,800 ± 412 µm^2 during the dry season, while that of the non-APHPs was set at 3,400 ± 809 µm^2 during the rainy season and at 5,200 ± 993 µm^2 during the dry season (Table 1, Figure 5).

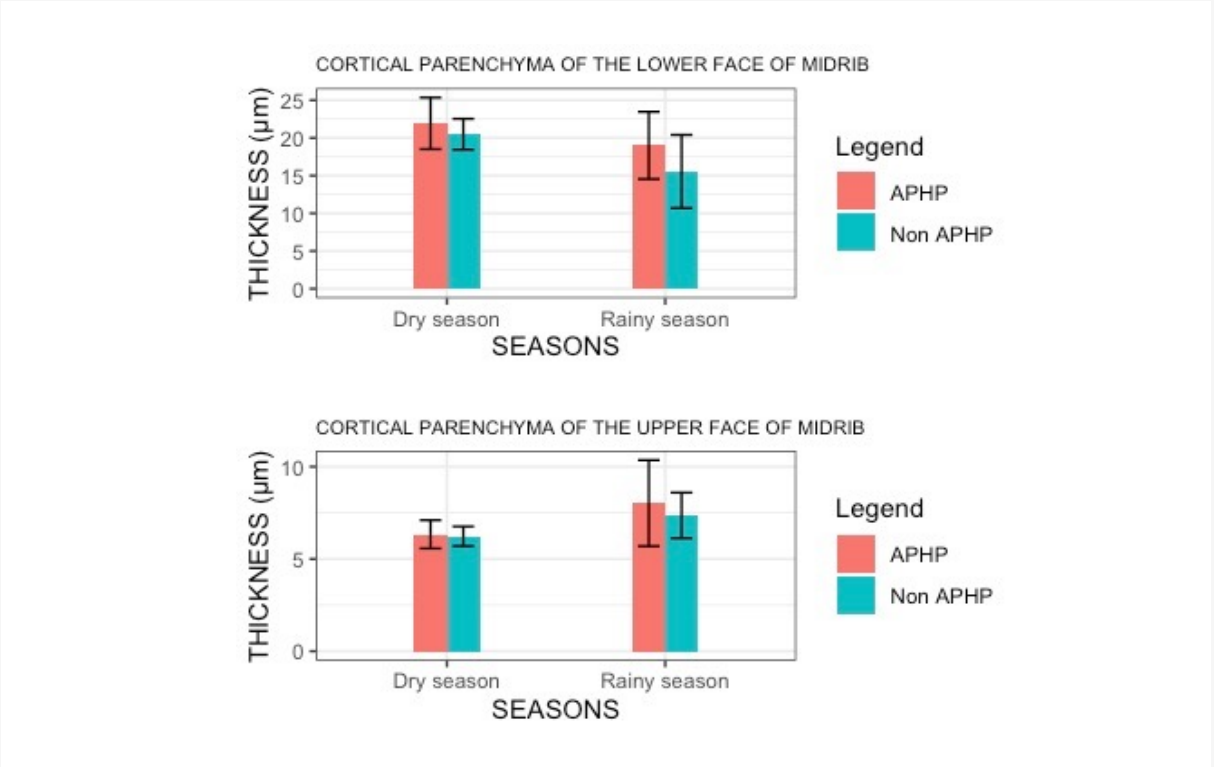

Figure 4. Thickness of cortical parenchyma.

Slika 4. Debelina kortikalnega parenhima.

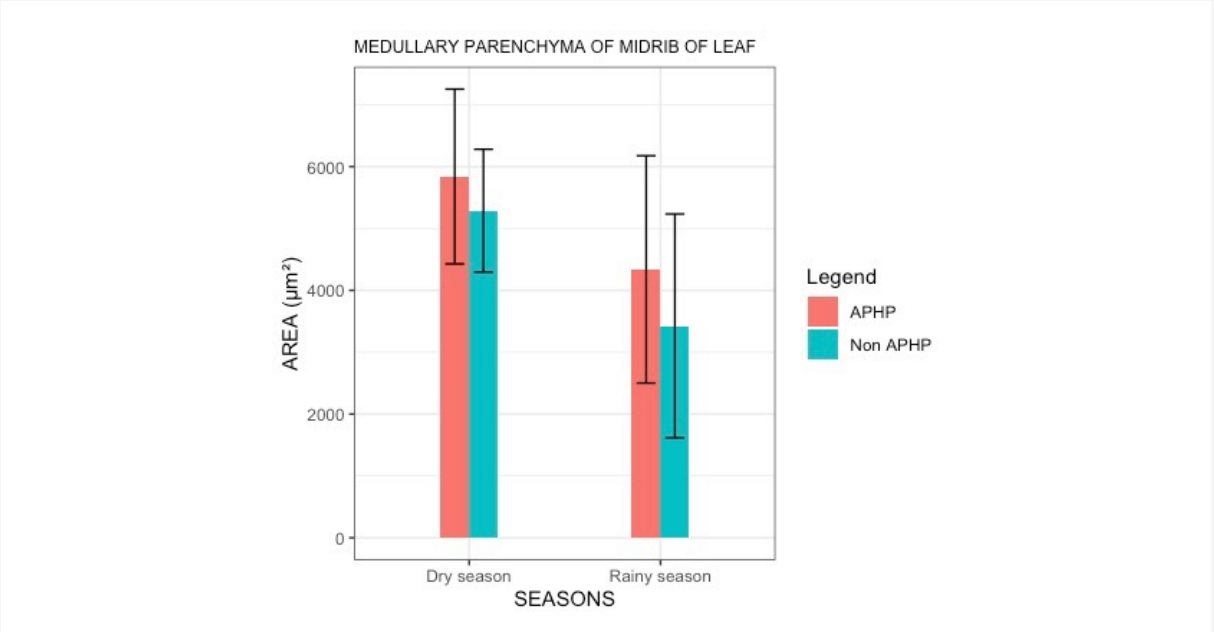

Figure 5. Area of the medullary parenchyma.

Slika 5. Območje medularnega parenhima.

Epidermis

Firstly, both APHPs and non-APHPs showed a similar thickness of the upper and the lower epidermis, and secondly, an upper epidermis thicker than the lower epidermis, independently of the seasons (Table 1, Figure 6). Moreover, both categories of cashew leaves showed an increase ($P \leq 0.036$) in the thickness of both the lower and upper epidermis from the rainy season to the dry season (Table 1, Figure 6).

Xylem

Both categories of cashew leaves showed the same thickness of xylem vessels for each season but a variation of the thickness of xylem vessels according to the seasons (Table 1, Figures 7 and 8). Indeed, there was a decrease ($P = 0.040$) in the non-APHPs' xylem vessel thickness which moved from 6.7 ± 0.9 µm during the rainy season to 5.9 ± 0.6 µm during the dry season, and a high decrease ($P = 0.004$) of the APHPs' xylem vessel thickness which changed from 6.5 ± 0.7 µm during the rainy season to 6.4 ± 0.5 µm the dry season (Table 1, Figure 7).

Stomatal density and opening surface

The average stomatal density at the abaxial faces of the leaves was the same ($P \geq 0.365$) for the APHPs and the non-APHPs during each season. However, the average stomatal density of APHPs was lower, with 155 ± 16.9 stomata/mm2 during the rainy season than those of 190.4 ±41.6 stomata/mm2 during the dry season (Figure 9). The non-APHPs also showed a lower average stomatal density of 147 ± 15.6 stomata/mm2 during the rainy season than those of 170.4 ± 20.8 stomata/mm2 during the dry season (Figure 9).

In both APHP trees and non-APHP trees, water deficit affected ($P < 0.018$) the opening width of the stomata, reducing it from 59.8 µm to 23.0 µm and from 47.3 to 22.2 µm, respectively, during the dry season (Figure 10). During each season, the stomata opening width was the same ($P > 0.552$) for the APHPs and the non-APHPs, which was 35.5 µm during the rainy season (Figure 11) and 19.2 µm during the dry season (Figure 12).

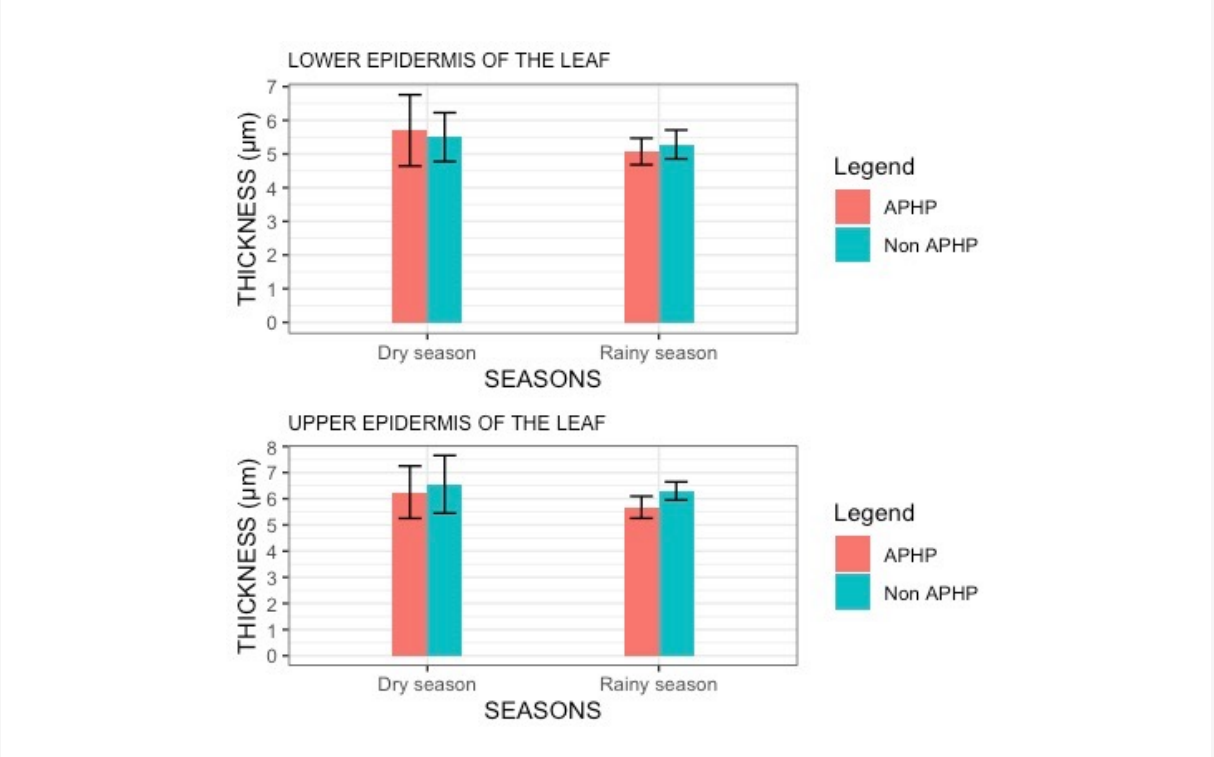

Figure 6. Thickness of the upper and lower leaf epidermis according to the season.

Slika 6. Debelina zgornje in spodnje listne povrhnjice glede na sezono.

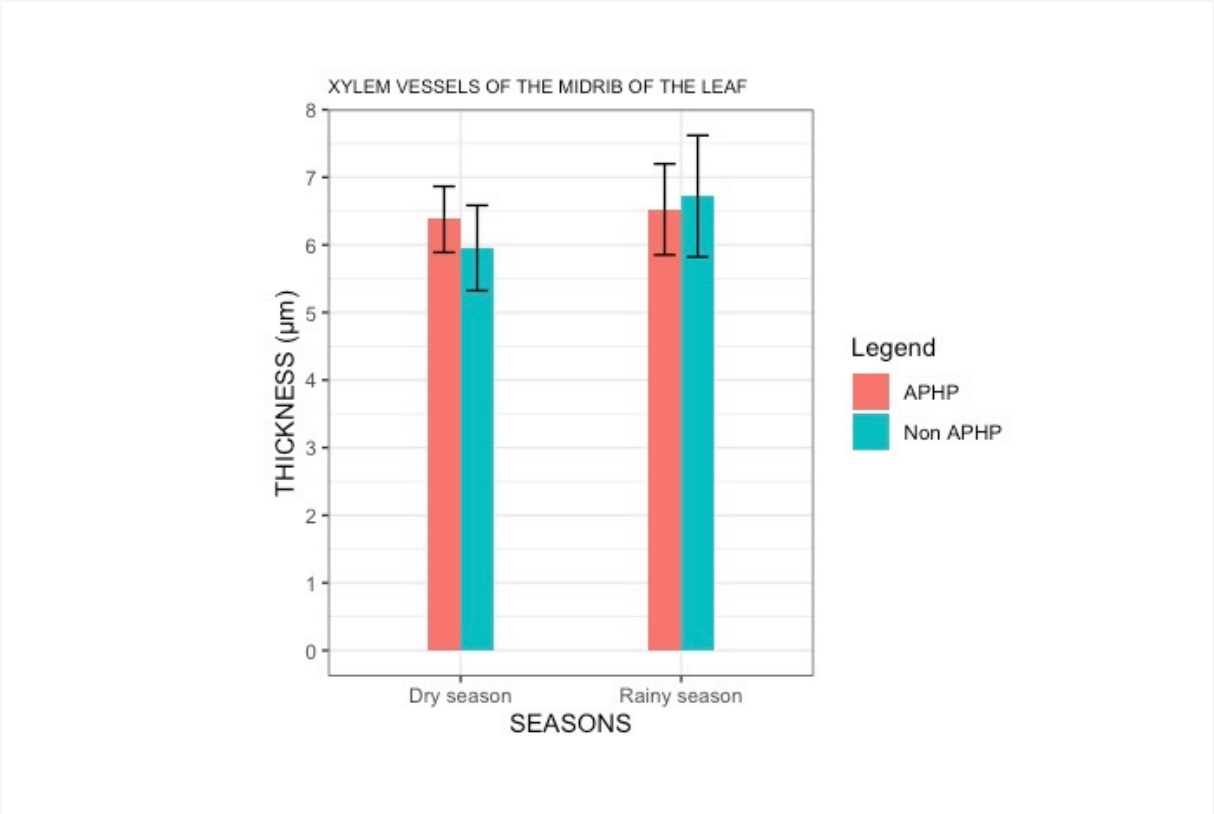

Figure 7. Xylem vessel thicknesses of the leaf midrib.

Slika 7. Debelina ksilemskih žil na sredini lista.

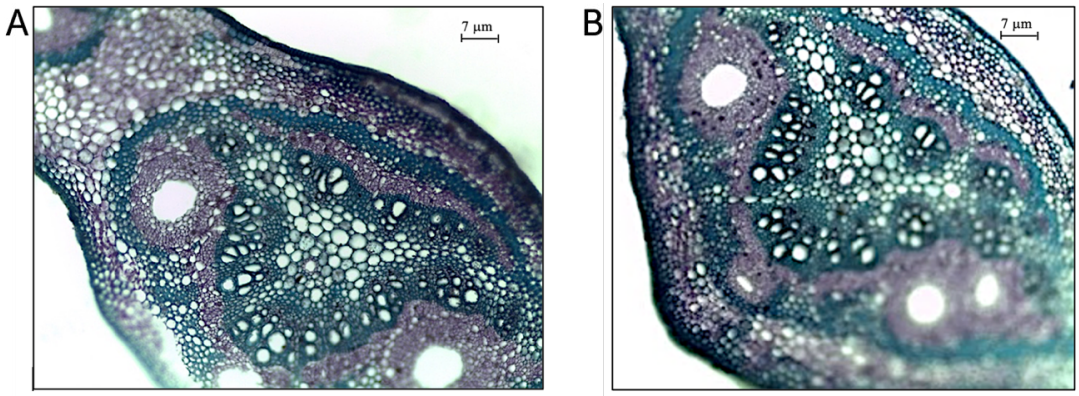

Figure 8. The anatomical structure of the main vein of cashew leaves according to the season (40× magnification). A: during the rainy season (production); B: during the dry season (after production).

Slika 8. Anatomska zgradba glavne žile listov indijskega oreščka glede na sezono (40-kratna povečava). A: v deževnem obdobju (proizvodnja); B: v suhem obdobju (po proizvodnji).

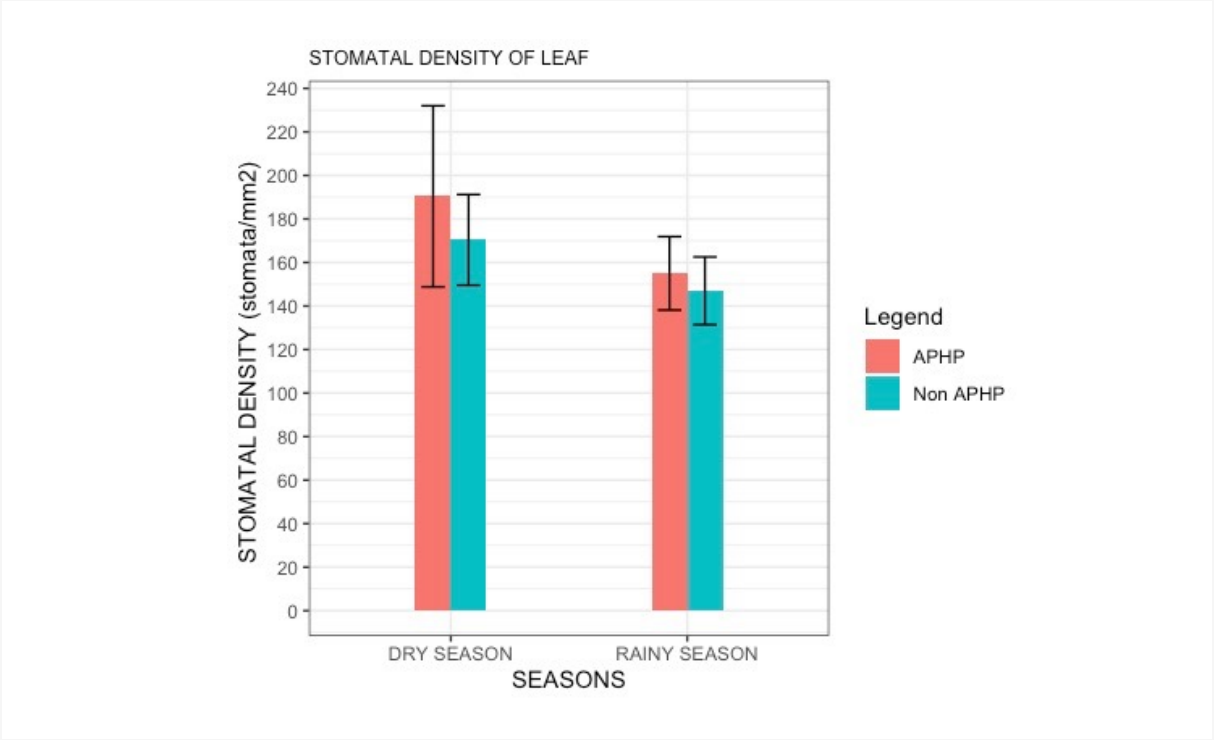

Figure 9. The stomatal density of cashew trees. For each locality of origin of the cashew trees, the letter V is used to distinguish the non-APHPs from the APHPs.

Slika 9. Stomatalna gostota indijskih dreves. Za vsako lokacijo izvora indijskih dreves je uporabljena črka V za razlikovanje med drevesi, ki niso APHP, in drevesi, ki niso APHP.

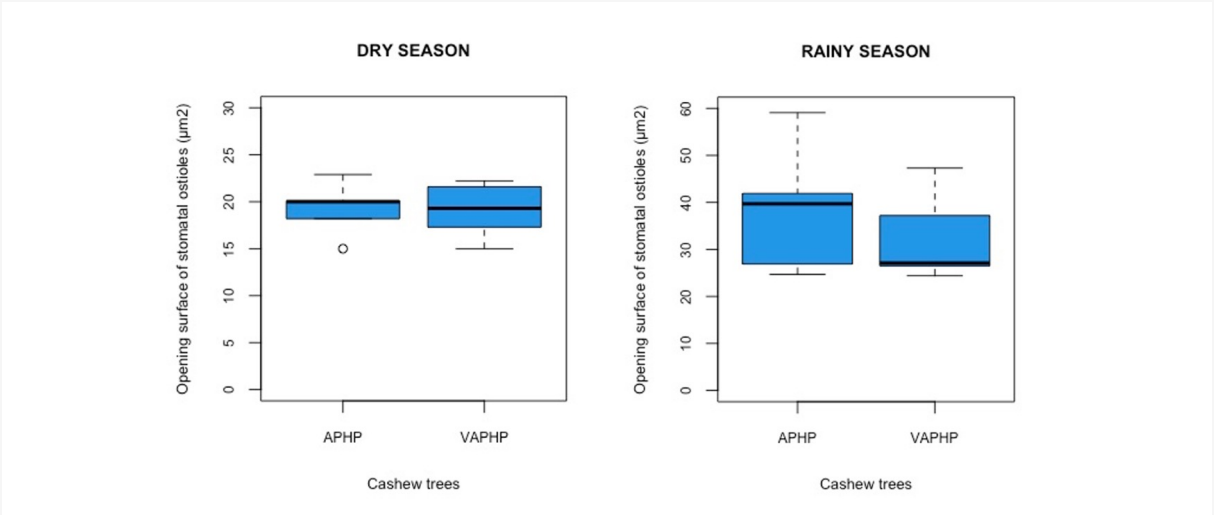

Figure 10. Variation in the surface area of cashew tree stomata ostioles depends on the seasons. For each locality of origin of the cashew trees, the letter V is used to distinguish the non-APHPs from the APHPs.

Slika 10. Razlike v površini ostiol listnih rež indijskega drevesa glede na letni čas. Za vsako lokacijo izvora indijskih dreves je uporabljena črka V za razlikovanje med drevesi, ki niso APHP, in drevesi, ki niso APHP.

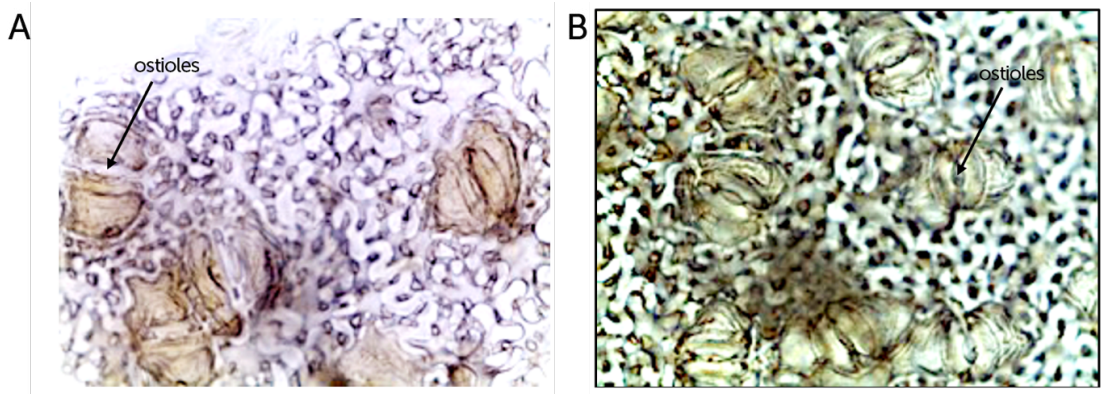

Figure 11. Surface areas of stomatal ostioles during the rainy season. A: APHP; B: non-APHP (100× magnification).

Slika 11. Površine stomatalnih ostiol v deževnem obdobju. A: APHP; B: brez APHP (100-kratna povečava).

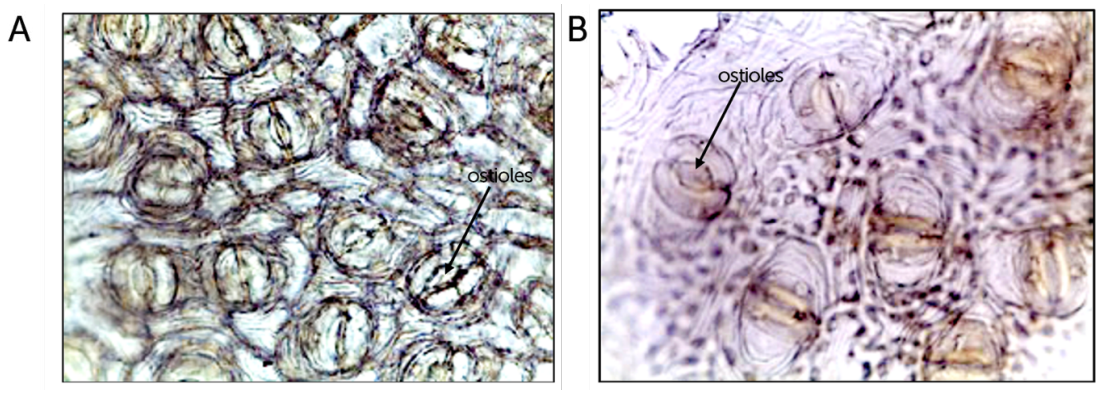

Figure 12. Surface areas of stomatal ostioles during the dry season. A: APHP; B: non-APHP (100× magnification).

Slika 12. Površina stomatalnih ostiolov v sušnem obdobju. A: APHP; B: brez APHP (povečava 100×).

Root tissues

Xylems

The root xylems were characterized by both their vessel number and diameter.

The APHPs' root xylem vessel number varied between 11 and 26 vessels/mm2, while that of the non-APHPs varied between 15 and 42 vessels/mm2 (Figure 13). ANOVA did not show ($P = 0.051$) any impact of the season on the number of xylem vessels in both APHP and non-APHP roots.

The largest root xylem radial diameters, set between 16.62-18.62 µm and tangential diameters set between 13.02-16.92 µm, were found during the rainy season (Figure 14), while the smallest radial diameters, about 4.88 µm and tangential diameters about 3.48 µm were found during the dry season (Figure 15). However, no impact of the season ($P = 0.263$) was found on the root xylem vessel diameter of both APHPs and non-APHP cashew trees.

No correlation ($P > 0.380$) was found between the number of xylem vessels and the ratio of the tangential and radial diameters of the vessels of the roots of both APHPs and non-APHPs.

For each locality of origin of the cashew tree, the letter V is used to distinguish the non-APHPs from the APHPs.

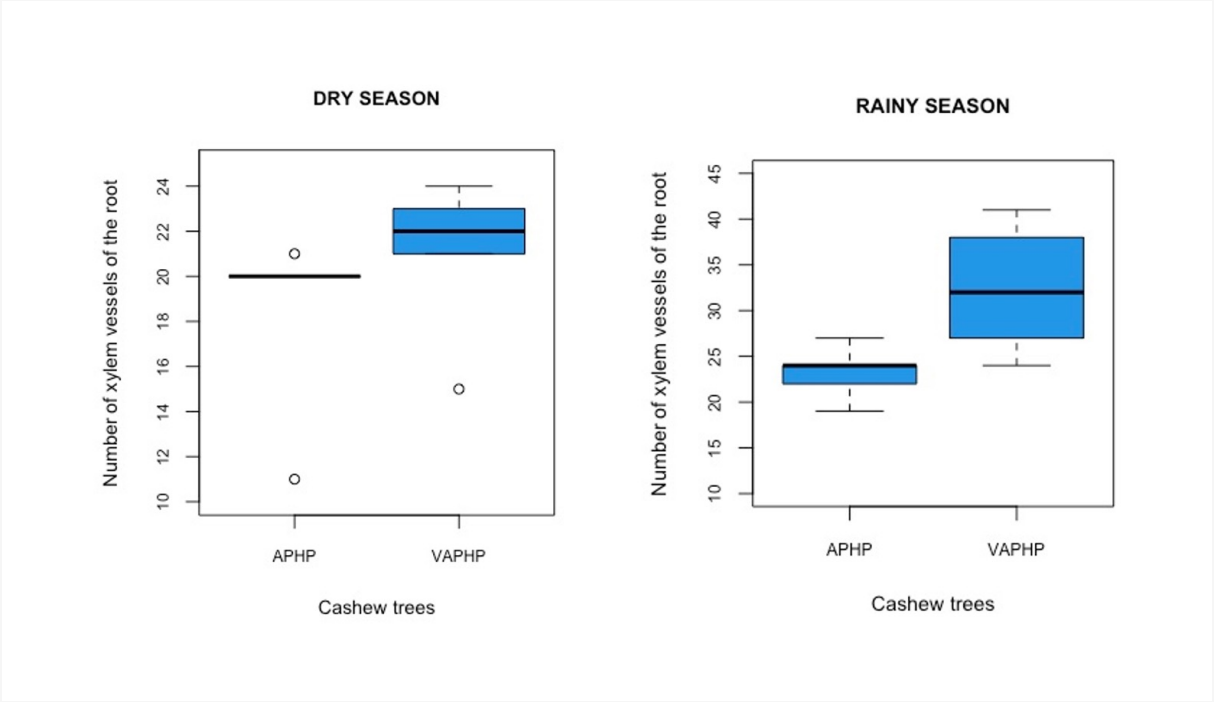

Figure 13. The number of xylem vessels in the cashew tree roots according to the cashew categories and the season.

Slika 13. Število ksilemskih žil v koreninah indijskega drevesa glede na kategorije indijskega oreščka in sezono.

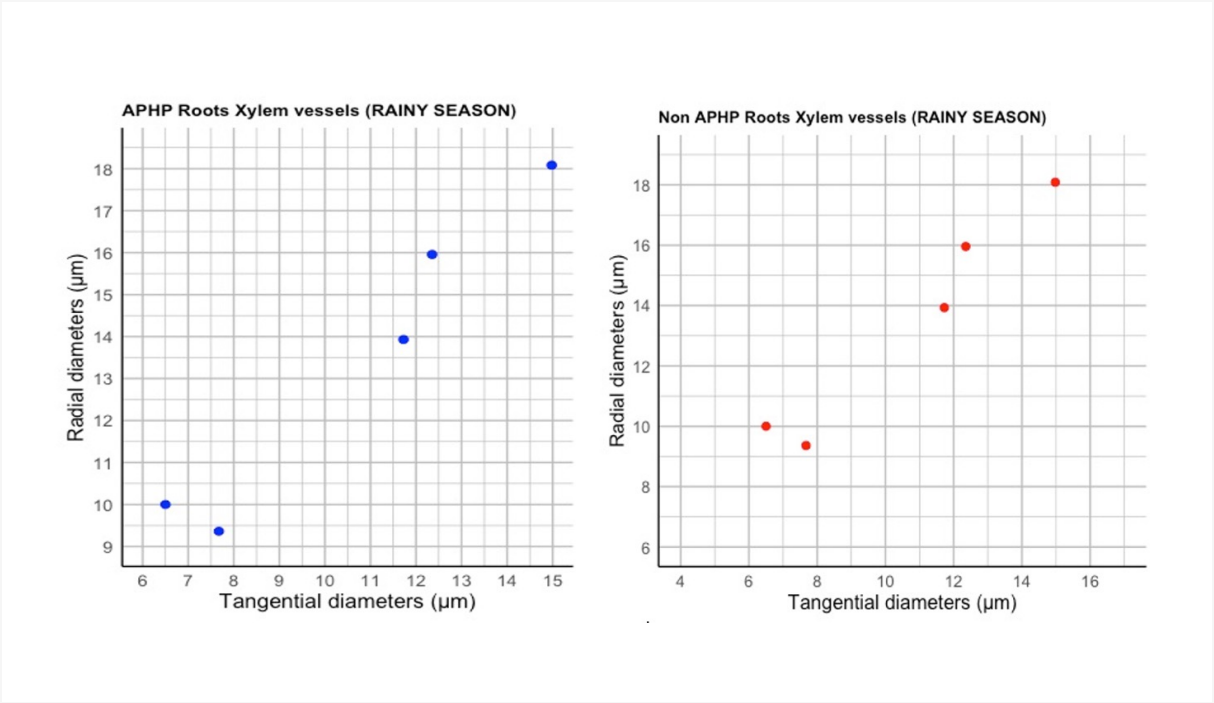

Figure 14. Tangential and radial diameters of the xylem vessels of cashew trees during the rainy season.

Slika 14. Tangencialni in radialni premeri ksilemskih žil indijskih dreves med deževno sezono.

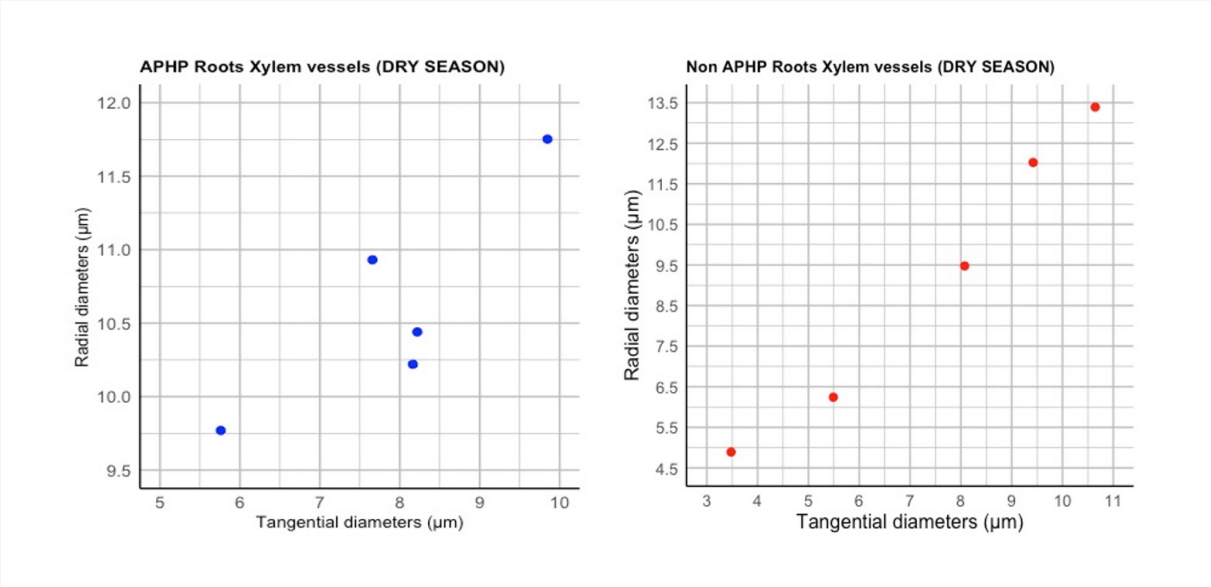

Figure 15. Tangential and radial diameters of the xylem vessels of cashew trees during the dry season.

Slika 15. Tangencialni in radialni premeri ksilemskih žil indijskih dreves v sušnem obdobju.

Medullar parenchyma

The average thickness of the roots medullary parenchyma increased with water deficit (Figure 16). The APHPs and non-APHPs experienced average medullary parenchyma thicknesses of 70 µm and 62 µm, respectively, during the dry season (Figure 16), while these values were about 44 µm for the APHPs and 20 µm for the non-APHP (Figure 17). Independently of the season, the APHPs showed a larger ($P = 0.003$) average medullary parenchyma thickness of roots medullar parenchyma than the non-APHPs (Figures 16 and 17).

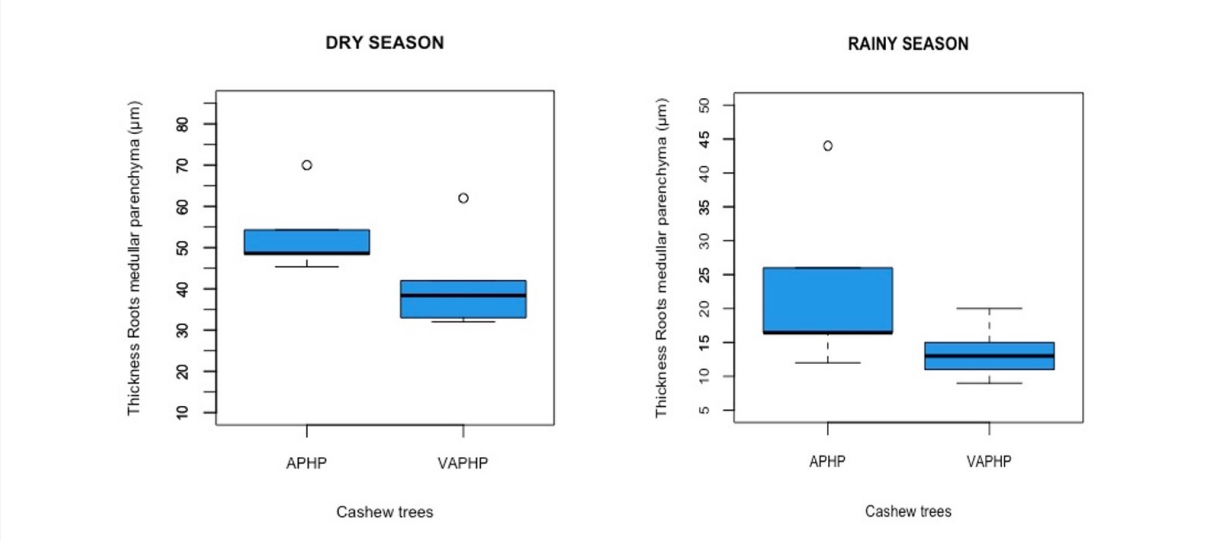

Figure 16. Thickness of the medullary parenchyma of the cashew tree roots. The letter V is used to distinguish the non-APHPs from the APHPs.

Slika 16. Debelina medularnega parenhima korenin indijskega drevesa. Črka V je uporabljena za razlikovanje med ne-APHP in APHP.

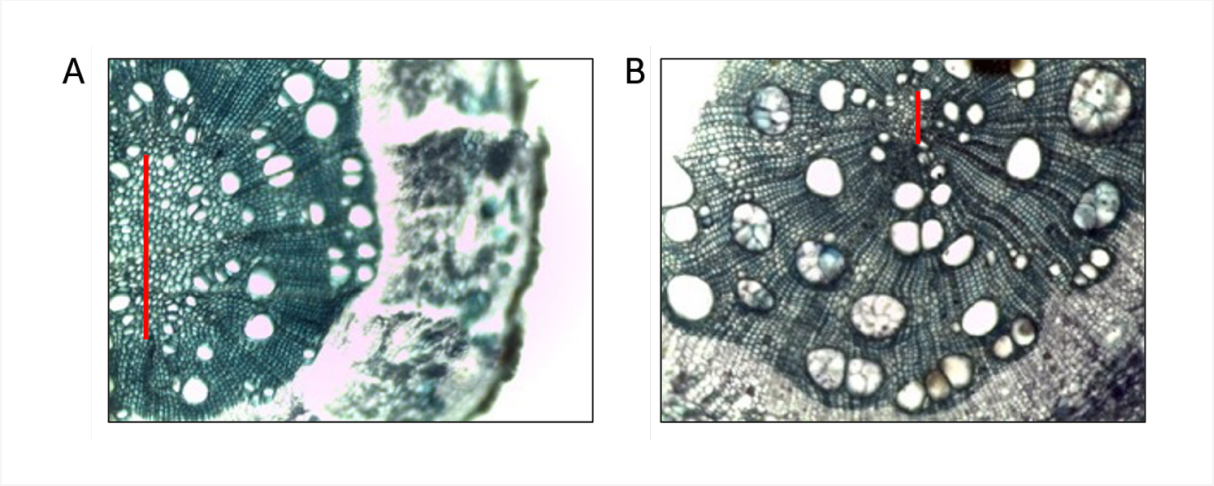

Figure 17. Roots medullary parenchyma width during the dry season (A) and the rainy season (B).

Slika 17. Širina medularnega parenhima korenin v sušnem obdobju (A) in deževnem obdobju (B).

Discussion

Plants exposed to water deficits undergo biochemical changes as well as various anatomical adaptations, enabling them to grow under stress conditions (Saeed et al. 2016). In this study, water deficit conditions during the dry season significantly increased the cortical parenchyma of the main vein from 18.5 ± 4.5 µm to 22 ± 3.4 µm. This may indicate that cashew trees adapt their anatomical structure to water-limited environments. As Boughalleb et al. (2014) demonstrated in another study on *Astragalus gombo* Coss. & Durieu ex Bunge subsp. *gomboeformis* (Pomel) Ott, various anatomical adaptations, including cortical and mesophyll parenchyma formation, occur in response to limited water availability. These features are suggested to contribute to maintaining water potential and storing energy during drought, which effectively enhances plant survival in arid environments.

A significant increase was also observed in leaf anatomical characteristics, such as the thickness of the palisade parenchyma on both surfaces (upper surface: 54 ± 4.2 µm to 59 ± 5.5 µm, lower surface: 20 µm ± 2.9 µm to 28 ± 2.1 µm) and in the thickness of the medullary parenchyma (from 4300 ± 837.7 μm^2 to 5800 ± 412.7 μm^2) of the main vein during the dry season. These findings do not align with Akram et al. (2016), who reported that water stress in radish cultivars significantly reduced the main vein parenchyma thickness. Previous studies have shown that water stress can considerably reduce leaf mesophyll thickness and main vein thickness in *Triticum aestivum* L. (Burnett et al. 2005) and *Ctenanthe setosa* (Roscoe) Eichler (Kutlu et al. 2009). Similarly, Bosabalidis and Kofidis (2002) demonstrated that severe stress negatively affects mesophyll and palisade parenchyma thickness. Our findings also revealed a decrease in cashew lacunar parenchyma, regardless of production potential (Figure 5). Similarly, Fernández et al. (1997) found that water deficit in *Olea europaea* L. reduces mesophyll intercellular spaces, causing morphological and anatomical modifications in leaves.

The reduction in epidermal cell thickness is an adaptive strategy that contributes to leaf resistance against cellular collapse due to drought and controls transpiration at the cuticle level (Bosabalidis and Kofidis 2002). However, in the cashew leaves, the epidermal cell size increased from 5.6 ± 0.4 µm during the rainy season to 6.2 ± 1.0 µm during the dry season, thus reducing water loss as stomatal density increased (Figure 9).

Ennajeh et al. (2010) also observed that the lacunar parenchyma thickness is higher under water deficit. This increased thickness during the dry season in olives is attributed to an enlargement of intercellular spaces, which enhances CO_2 diffusion.

The larger thickness of the xylem in the main leaf vein during the rainy season (6.7 ± 0.9 µm) than in the dry season (5.9 ± 0.6 µm) can likely be due to greater water availability in the soil. This water availability increases hydraulic conduc-

tivity, which increases xylem thickness and vascular bundle number. Similar results were found in roots, with both vessel number (19.7 ± 3.9 µm to 27.8 ± 7.1 µm) and vessel thickness (radial diameter: 16.6-18.6 µm to 4.9 µm; tangential diameter: 13.0-16.9 µm to 3.5 µm) increasing during the rainy season. This strategy is also adopted by grapevine cultivars, which respond to water limitations by adopting specific hydraulic strategies (Lovisolo et al. 2010; Dal Santo et al. 2016). Moreover, the formation of smaller vessels under drought conditions has been observed in the stems of various tree species, such as *Quercus* L. and *Populus* L. (Arend and Fromm 2007, Fonti et al. 2013). Small vessels are suggested to be less vulnerable to xylem embolism induced by drought and contribute to water flow regulation under water-limited growth conditions. Additionally, the anatomical response to water limitation in grapevines includes adaptation through modification of xylem traits (xylem conduit size and number). Conversely, the increase in xylem vessel number and thickness enhances cavitation tolerance, conferring drought resistance (Arend and Fromm 2007). Smaller vessel diameters facilitate hydraulic conductance, reduce cavitation, and make water and nutrient uptake more efficient (Queiroz-Voltan et al., 2014). Under water stress, smaller xylem vessels promote efficient and safer water transport, improving hydraulic conductivity (Batista et al. 2010). In contrast, larger xylem vessels are more common in areas with higher rainfall for a given species (Mauseth 1988). The olive tree, a drought-resistant species, reduces xylem vessel diameter under water stress, allowing the plant to maintain transpiration flux (Ben et al. 2007).

Our findings indicate that stomatal density in cashew trees ranges from 125 to 259 stomata per mm^2 (Figure 9). These results align with Esau (1965), who reported a density range of 100-300 stomata per mm^2 in numerous plant species. Stomata are denser during the dry season (259 stomata/mm^2) than the rainy season (176 stomata/mm^2). The stomatal aperture is smaller in the dry season (23 µm) than in the rainy season (59.3 µm), consistent with Ma (2010), who found that increased osmotic pressure in guard cell vacuoles, following high solute absorption, induces stomatal pore opening. Raven et al. (2013) observed that under hot, dry conditions, plants enter water stress, triggering the production of abscisic acid, a regulatory hormone secreted by leaves and roots that participates in various physiological regulation mechanisms. Under drought conditions, abscisic acid synthesis increases in the roots, where it is transported to the leaves to initiate prolonged stomatal closure (William 1995). Abscisic acid binds to its receptor in the plasma membrane, causing a calcium-mediated phosphorylation cascade that releases ions and leads to guard cell turgor loss, reducing stomatal opening. Thus, decreased stomatal aperture during osmotic stress is the most pronounced response (Munns and Tester 2008).

In several fruit tree species, the response to water deficit involves reducing stomatal area, as seen in pistachio trees (Abbaspour et al. 2012), other Mediterranean species (Ben Ahmed et al. 2008), and the *Olea europaea* L. as found in Guerfel et al. (2009). This mechanism limits CO_2 assimilation during water deficits. Fernández et al. (1997) also observed that reduced CO_2 assimilation may decrease leaf mesophyll conductance.

Moreover, the highest stomatal densities were observed in cashew trees during the dry season. Grisi et al. (2008) reported similar results for irrigated and non-irrigated coffee plants, with non-irrigated plants showing higher stomatal density. Similarly, Batista et al. (2010) showed that higher stomatal densities observed under water deficit in coffee plants indicate greater drought tolerance. Plants, being sessile, must adapt to various environmental factors, with stomata playing a crucial role in this function (Casson and Gray 2008). This supports the existence of drought resistance characteristics in non-irrigated coffee plants with higher stomatal density.

Conclusion

The conditions impacted leaf and root tissues. The thickness of the palisade parenchyma of non-APHPs remained constant, while that of APHPs increased considerably from the rainy season to the dry season. The two types of trees had the same reaction to water deficit, which was the increase in upper and lower epidermis thickness in order to reduce water loss during the dry season. During the dry season, the thickness of the xylem decreased, the average thickness of the cortical parenchyma of the lower midrib increased, and the average thickness of the cortical parenchyma of the lower midrib decreased, the thickness of the intercellular spaces of the lacunous parenchyma decreased, and the thickness of the medullary parenchyma of the midrib of the leaves of APHPs and their neighbours increased. The stomatal density increased during the dry season in all the cashew trees. However, the opening surface of the stomatal ostioles decreased during the dry season. These results

showed the different anatomical adaptation strategies of the cashew tree to water constraints.

Authors' contributions

Conceptualization, K.A.R.F., K.M.L., and K.N.F.; methodology, K.A.R.F., K.M.L., and K.N.F.; software, K.A.R.F.; validation, K.M.L., and K.N.F.; formal analysis, K.A.R.F.; investigation, K.A.R.F. ; resources, K.A.R.F. ; data curation, K.A.R.F. ; writing—original draft preparation, K.A.R.F. ; writing—review, and editing, K.M.L., and K.N.F.; visualization, K.A.R.F. , and K.M.L.; supervision, K.N.F.; project administration, K.N.F.; funding acquisition, K.N.F.. All authors have read, and agreed to the published version of the manuscript.

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Data availability

The authors confirm that the datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of interest

No potential conflict of interest was reported by the authors.

References

Abbaspour, H., Afshari, H., Abdel-Wahhab, A., 2012. Influence of salt stress on growth, pigments, soluble sugars and ion accumulation in three pistachio cultivars. In: J. Med. Plant. Res., 6, 2468-2473. https://doi.org/10.5897/JMPR11.1710

Akram, N.A., Shafiq, S., Ashraf, M., Aisha, R., Sajid, M.A., 2016. Drought- induced anatomical changes in radish (*Raphanus sativus* L.) leaves supplied with trehalose through different modes. Arid Land Res. Manag., 30(4), 412–420. https://doi.org/10.1080/15324982.2016.1145760

APG., 2016. An update of the Angiosperm Phylogeny Group classifcation for the orders and families of fowering plants, APG IV. Bot. J. Linn. Soc., 181, 1-20. https://doi.org/10.1111/boj.12385

Arend, M., Fromm, J., 2007. Seasonal change in the drought response of wood cell development in poplar, Tree Physiol., 27, 985-992. https://doi.org/10.1093/treephys/27.7.985

Batista, L.A., Guimarães, R.J., Pereira, F.J., Carvalho, G.R., Castro, E.M., 2010. Leaf anatomy and water potential in the tolerance of coffee cultivars to water stress. Sciences agricoles, Fortaleza, 41(3), 475-481. [Portuguese]. http://dx.doi.org/10.1590/S1806-66902010000300022

Ben Ahmed, H., Manaa, A., Zid, E., 2008. Salinity tolerance of a short-cycle Poaceae: foxtail (Setaria verticillata L.) Biol. Rep., 331(2), 164-170. [French]. https://doi.org/10.1016/j.crvi.2007.12.002

Ben, R.B., D'Andria, R. Trigui, A., Boukhris, M. ,Chaïeb, M., 2007. Effects of Water Stress and Soil Type on Photosynthesis, Leaf Water Potential, and Yield of Olive (Olea europaea L. cv. Chemlali Sfax). Aus. J. Exp. Agriculture, 47(12), 1484-1490. https://doi.org/10.1071/EA05206

Bosabalidis, A., Kofidis, G., 2002. Comparative effects of drought stress on leaf anatomy of two olive cultivars, Plant Sci., 163, 375-379. https://doi.org/10.1016/S0168-9452(02)00135-8

Boughalleb, F., Abdellaoui, R., Brahim, N., Neffati, M., 2014. Anatomical Adaptation of *Astragalus gombiforme* Head subjected to drought stress, Cent. Eur. J. Biol., 12, 1215-1225. [French] https://doi.org/10.2478/s11535-014-0353-7

Burnett, S.E., Pennisi, S.V., Thomas, P.A., Marc W.V.L., 2005. Controlled drought affects the morphology and anatomy of *Salvia splendens*. J. Am. Soc. Hort. Sci., 130, 775-781. https://doi.org/10.21273/JASHS.130.5.775

Casson, S., Gray, J.E., 2008. Influence of environmental factors on stomatal development. New Phytol., 178(1), 9-23. https://doi.org/10.1111/j.1469-8137.2007.02351.x

Charahabil, M.M., Diatta, M., Ndiaye, S., 2017. Characterization of cashew plantations (*Anacardium occidentale* L.) in Balantacounda: case of the communes of Kaour, Goudomp and Djibanar (Casamance/Senegal). J. Euro. Sci., 242-257. https://doi.org/https://10.19044/esj.2017.v13n12p242

Cramer, G., Urano, K., Delrot, S., Pezzotti, Mario., Shinozaki, K., 2011. Effects of abiotic stress on plants: a systems biology perspective, BMC Plant Biol., 11, 163. https://doi.org/10.1186/1471-2229-11-163

Dal Santo, S., Palliotti, A., Zenoni, S., Tornielli, G.B., Fasoli, M., Paci, P., Tombesi, S., Frioni, T., Silvestroni, O., Bellincontro, A., 2016. Distinct transcriptome responses to water limitation in isohydric and anisohydric grape cultivars. Génome BMC. 17 , 815 p. https://doi.org/10.1186/s12864-016-3136-x

Djaha, A.J.B., N'guessan, A.K., Ballo, C.K., Aké, S., 2010. Seeds of two elite varieties of cashew tree (*Anacardium occidentale* L.) intended to serve as rootstock in Côte d'Ivoire. J. App. Biosci., 32, 1995-2001. [French].

Djinet, A.I., Bell, J.M., Nana, R., Nguinambaye, M.M., Tamini, Z., 2016. Evaluation of stomata characteristics in oil palm (*Elaeis guineensis* Jacq.). J. App. Biosci., 104, 99 4-9910. [French]. https://doi.org/10.4314/jab.v104i1.2

Ennajeh, M., Vadel, A., Cochard, H., Khemira, H., 2010. Comparative impacts of water stress on the leaf anatomy of a drought-resistant and a drought-sensitive olive cultivar. J. Hort. Sci. Biotechnol., 85, 289-294. https://doi.org/10.1080/14620316.2010.11512670

Esau, K., 1965. Anatomy of seed plants. John Wiley ed. New York, USA, 549 pp.

FAO, 2005. Agroecological zone of Côte d'Ivoire. Irrigation in Africa in figures. Aquastat survey. http://www.fao.org/nr/water/aquastat/countries_regions/civ. (08/08/2023, date last accessed).

FAO, 2024. Cashew nut production. https:// www.fao.org/faosta t/fr/#da ta /qcl. (consulted on 05/04/2024). [French].

Fernández, J.E., Moreno, F., Giron, I.F., Blazquez, O.M., 1997. Stomatal control of water use in olive leaves. Plant Soil, 190, 179-192. https://doi.org/10.1023/A:1004293026973

Fonti, P., Heller, O., Cherubini, P., Rigling, A., Arend, M., 2013. Wood anatomical responses of oak saplings exposed to air warming and soil drought. Plant Biol., 15, 210-219. https://doi.org/10.1111/j.1438-8677.2012.00599.x

Grisi, F.A., José, D.A., Evaristo, M.C., Cynthia, O., Gabriel, B., Lucas, A.M., 2008. Leaf anatomical evaluations of 'Catuaí' and 'Siriema' coffee seedlings subjected to water stress. Science and Agrotechnology, Lavras, 32(6), 1730-1736. [Portuguese]. https://doi.org/10.1590/S1413-70542008000600008

Guerfel, M., Baccouri, O., Boujnah, D., Chaibi, W., Zarrouk, M., 2009. Impacts of water stress on gas exchange, water relations, chlorophyll content and leaf structure in the two main Tunisian olive (*Olea europaea* L.) cultivars. Sci. Hort., 119, 257-263. https://doi.org/10.1016/j.scienta.2008.08.006

Grishaguin, I.V., 1985. Automatic cell counting with Image J, Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, 19 pp. https://doi.org/10.1016/j.ab.2014.12.007

Henry, G.A., Piperno, D., Brooks, A., 2011. Microfossils in calculus demonstrate consumption of plants and cooked foods in Neanderthal diets (Shanidar III, Iraq; Spy I and II, Belgium), Proc. Nat. Acad. Sci., 108(2), 486-491. https://doi.org/10.1073/pnas.1016868108

Kiwuso, P., Mujuni, D.B., Esegu, J., 2013. The Role of Social Capital in Building Shared Need for a Genuine Cause in Farmer Groups: Experience from Teso Organic Cashew Farmers Organization, International Cashew Conference, 119 pp.

Konan, A.D.S.H., 2016. Agro-Morphological Evaluation of Tall Cashew Trees (*Anacardium occidentale* (L.)) Identified in the Poro and Bagoue Regions. Master of Biotechnology, Biosecurity and Bioresource from Félix Houphouet-Boigny University, Abidjan, Côte d'Ivoire 83 pp. [French].

Konaté, M.L., 2021. Characterization of weeds in cashew orchards (*Anacardium occidentale* L., Anacardiaceae) in the cashew basin of Côte d'Ivoire. PhD thesis, Nangui Abrogoua University, 203 pp. [French].

Kutlu, N., Terzi, R., Tekeli, Ç., Senel, G., Battal, P., Kadioglu, A., 2009. Changes in anatomical structure and endogenous phytohormone levels during leaf rolling Ctenanthe setosa, Turk. J. Biol., 33, 115-122. https://doi.org/10.3906/biy-0806-6

Lovisolo, C., Perrone, I., Carra, A., Ferrandino, A., Flexas, J., Medrano, H., and Schubert, A., 2010. Drought-induced changes in grapevine (Vitis spp.) organ development and function and their hydraulic and non-hydraulic interactions at the whole-plant level: a physiological and molecular update, Fonction. Usine. Biol., 37, 98. https://doi.org/10.1071/FP09191

Ma, Z., 2010. Plant vacuoles and the regulation of stomatal opening. Nature Edu., 3(9), 45.

Marchi, S., Minnocci, A., Sebastiani, L., 2008. Variation in mesophyll anatomy and photosynthetic capacity during leaf development in a mesophytic deciduous fruit tree (*Prunus persica*) and an evergreen sclerophyll Mediterranean shrub (*Olea europaea* L.), Trees, 22,559-571. https://doi.org/10.1007/s00468-008-0216-9

Mauseth, J.D., 1988. Plant Anatomy. Benjamin/Cummings Publ. Co., Menlo Park.

Munns, R., Tester, M., 2008. Mechanisms of Salinity Tolerance. Ann. Rev. Plant Biol., 59, 651-681. https://doi.org/10.1146/annurev.arplant.59.032607.092911

Ouattara, D., Kouamé, D., Tiébré, M.S., Kouadio, Y.J.C., N'guessan K.E., 2016. Plant biodiversity and use value in the Sudanian zone of Ivory Coast, Int. J. Biol. Chem. Sci., 10(3), 1122-1138. [French]. https://doi.org/10.4314/ijbcs.v10i3.18

Queiroz-Voltan, R. B., Nardin, C.F., Fazuoli L.C., Masako, T.B., 2014. Characterization of the Leaf Anatomy of Arabica Coffee Plants in Different Seasonal Periods. Revista Biotemas, Florianópolis, 27(4), 1-10 [Portuguese]. https://doi.org/10.5007/2175-7925.2014v27n4p1

Raven, P.H., Evert, R.F., Eichhorn, S.E., 2013. Biology of Plants. 8th Edition. Freeman and Company Publishers, New-York, NY, USA. 727 pp. https://doi.org/10.1093/aob/mcu090

Ruzin, S., 1999. Plant microtechnology and microscopy, Oxford University Press, New York, 322 pp. https://doi.org/10.1046/j.1469-8137.2000.00735.x

Saeed, N., Maqbool, N., Haseeb, M., Sadiq, R., 2016. Morpho-anatomical changes in roots of chickpea (*Cicer arietium* L.) under drought stress condition, J. Agricult. Sci. Technol., 6, 1-9. https://doi.org/10.17265/2161-6264/2016.01.001

Sankharé, M., 2018. Phenotyping of cashew trees (*Anacardium occidentale* L.) from mass selection in the Fatick region (Senegal), Master's thesis: Cheikh Anta Diop University of Dakar (Senegal), 44 pp. [French].

Skirycz, A., Inze, D., 2010. More from less: plant growth under limited water, Cur. Opinion Biotechnol., 21, 197-203. https://doi.org/10.1016/j.copbio.2010.03.002

Stein, O., Avin-Wittenberg, T., Krahnert, I., Zemach, H., Bogol, V., Daron, O., Aloni, R., Fernie, A., Granot, D., 2017. *Arabidopsis* fructokinases are important for seed oil accumulation and vascular development, Fronti. Plant Sci., 7, 1-16. https://doi.org/10.3389/fpls.2016.02047

ABS

Original Research

Significant records of plants, algae, fungi, and animals in SE Europe and adjacent regions, 3

Aljaž Jakob [1], Laura Štampar [2], Žan Lobnik Cimerman [2], Simona Strgulc Krajšek [2], Miloš Vittori [2], Dren Dolničar [3]

Abstract

In this paper, we present two significant records of bryophytes in Slovenia, *Cryphaea heteromalla* and *Riccia cavernosa*, which were found in new phytogeographical regions. We report the three new records of an isopod *Armadillo officinalis*, a species that has been considered possibly extinct in Slovenia, and a new record of bat, *Plecotus macrobullaris*, from the Julian Alps.

Keywords

Cryphaea heteromalla; *Riccia cavernosa*; bryophytes; mosses, liverworts, flora; *Armadillo officinalis*; Isopoda; isopods; *Plecotus macrobullaris*; Chiroptera; bats; fauna; Slovenia

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Cryphaea heteromalla (Hedw.) D. Mohr., Crypheaceae (moss, bryophyte)

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Country	Slovenia
Statement of significance	First record in Slovenia outside the submediterranean region
Locality description	Gorenjska, Medvode, Gorenja vas-Reteče, the gravel pit 1.2 km northeast of the village.
Habitat	In a partially flooded gravel pit, a semi-permanently flooded riparian forest, on *Salix alba* bark
Date of observation	2024-06-28
Geographical coordinates	N 46.16747309°, E 14.38683831°
Voucher	Photo documented

Cryphaea heteromalla was hitherto known only from submediterranean parts in the southwestern part of Slovenia (Martinčič 2024). The localities are in Vipava Valley (Martinčič, 2016), from the area of Škocjan Caves (Strgulc Krajšek et al., 2023) and from the flysch Šavrini hills southeast of Koper, where the species is not rare in humid microlocalities (pers. obs.). The new record is the first find of this Atlantic-Mediterranean species (Hill et al., 2007) in the central part of Slovenia in the pre-Alpine phytogeographical region.

It has been noted this species is benefiting and spreading after the increase in air quality in Europe towards the end of 20th and beginning of 21st century. However, in Germany, the spread of this species has additionally been linked to climate change and eutrophication of the environment (Müller, 2016). Since the new locality is in a highly anthropogenic environment in intensely managed agricultural land, eutrophication and climate change might likely play a role in the spread of this species to continental parts of Slovenia. The species is expected to be found elsewhere in similar anthropogenic and eutrophic environments.

Cryphaea heteromalla is very distinctive when fertile, it has erect sporophyte-bearing shoots with several capsules growing along just one side (Figure 1). The capsules are 2.5–3 mm long and have a very short seta (Preston, 2010). In Europe, confusion is only possible with larger *Dendrocryphaea lamyana*, which grows in regularly flooded habitats, such as river rocks and bases of riparian trees, and has blunter leaves (Preston, 2010; Casas, 2006). It is a rare plant with strictly Atlantic-Mediterranean distribution, and it is not expected to occur in Slovenia (Hill et al., 2007).

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Figure 1. *Cryphaea heteromala* with characteristic erect shoots with several laterally growing capsules (photo: A. Jakob).

Riccia cavernosa (Hoffm.) Hoffm., fam. Ricciaceae (liverwort, bryophyte)

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Leg.	Laura Štampar
Country	Slovenia
Statement of significance	The third known locality in Slovenia, first in the dinaric phytogeographical region
Locality description	Notranjska, Cerkniško Jezero, Laze pri Gornjem Jezeru, left bank of river Jezerščica, next to the bridge near the Malnšče observation point, 550 m a.s.l.
Habitat	damp sandy soil
Date of observation	2024-08-30
Geographical coordinates	N 45.728611°, E 14.404167°
Voucher	Herbarium LJU (s.n.)

Riccia cavernosa (Hoffm.) Hoffm. is a temperate species of a complex thalloid liverwort with a circumpolar distribution. It is also present in the tropics and the Southern Hemisphere (Hill & Preston, 1998). It is typically found in temporarily wet habitats such as the banks of ponds, ditches, rivers, wetlands, and other periodically inundated sites (Frey et al., 2006).

R. cavernosa is a species from subgenus subgenus *Ricciella* (A. Braun) Boulay, section Spongodes Nees (Hodgetts et al., 2020). It is characterised by very distinctly perforated, yellow-green thallus without reddish colouration. A short, inconspicuous groove is visible on the upper surface only at the tip of the lobes (Long, 2010). The species has significant morphological similarities to *R. crystallina* L., therefore ripe spores are needed for reliable identification (Paton, 1999).

R. cavernosa is listed as the least concerned (LC) in the European Red List (Hodgetts et al., 2019). It is widespread in Europe but rare in the Mediterranean region (Blockeel et al., 2014), and its conservation status varies significantly across different countries. In Italy and Austria, it is endangered (EN), in Hungary, it is near threatened (NT), and in Croatia, it has no conservation status (Hodgetts and Lockhart, 2020). In Slovenia, it was found for the first time in 2021 in the vicinity of Maribor by Ž. Lobnik Cimerman (Ellis et al., 2022) and later also near Dokleževje in the Prekmurje Region (Lobnik Cimerman et al., 2023). In Slovenia, it has a status of endangered species (Martinčič, 2024).

Our sample was collected from a sandy substrate on the left bank of the Jezerščica River, approximately 80 meters downstream from the bridge near the Malnšče observation point. The Jezerščica River is part of the Stržen River, flowing near Gorenje Jezero, in the vicinity of intermittent Cerknica Lake. The Stržen is one of the main streams of the lake, flowing through this karst region and frequently forming seasonal floodplains. The banks of the Stržen River host numerous other plant species, mainly amphibious macrophytes, due to the constant water level fluctuation, creating a dynamic environment ideal for their growth. Some of the species of amphibious macrophytes with the highest abundance, which had already been recorded by Kržič et al. (2007), and were confirmed by our observations, are *Myosotis scorpioides, Nuphar luteum, Ranunculus trichophyllus, Rorippa amphibia* and *Schoenoplectus lacustris*.

Riccia cavernosa appeared in considerable abundance at the locality, covering entire sections of the riverbank (Figure 2), which is characteristic of the species when water level drops and its ecological requirements are met. Some field observations suggest that its spores can remain viable for several years (Blockeel et al., 2014). It appears that the current conditions are favourable for its growth, as the species is well adapted to fluctuations in water levels, typical of the area around Cerknica Lake and the Stržen River. However, excessive overgrowth of the riverbank could potentially threaten *R. cavernosa*.

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Figure 2. *Riccia cavernosa* growing on humid sandy soil (photo: L. Štampar).

Armadillo officinalis Duméril, 1816, fam. Armadillidae (animal)

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Country	Slovenia
Statement of significance	New records of a species considered possibly extinct in Slovenia
Locality description	(1) Sečovlje, Fontanigge, elevation 2 m.a.s.l. (2) Piran, by the Walls of Piran, elevation 35 m.a.s.l. (3) Sečovlje, Fontanigge, by the Museum of Salt Making, elevation 2 m.a.s.l.
Habitat	(1) Mediterranean halophilous scrub (2) Paved street (3) Mediterranean halophilous scrub
Date of observation	(1) 2023-09-21 (2) 2023-09-23 (3) 2024-03-30
Geographical coordinates	(1) N 45.47202°, E 13.6034° (2) N 45.52688°, E 13.57058° (3) N 45.47636°, E 13.59347°
Voucher	Six specimens collected in Piran are kept in the isopod collection of Miloš Vittori, University of Ljubljana, Biotechnical Faculty, Department of Biology, Večna pot 111, 1000 Ljubljana, Slovenia. Individuals observed at Fontanigge were not collected and were photographed instead.

The pill bug *Armadillo officinalis* Duméril, 1816 was first recorded on the territory of Slovenia in Škocjan near Koper in July 1926 (Strouhal, 1929). This record was the basis for the inclusion of the species in checklists of the Slovenian terrestrial isopod fauna (Potočnik, 1989; Vittori et al., 2023), but the species was not recorded in Slovenia again for nearly a century. Investigations of the terrestrial isopod fauna of the Slovenian coast in the 1970s and 1980s did not detect *A. officinalis* (Potočnik, 1984). As a result, the species was first considered endangered (Potočnik, 1992), and later, as efforts to find it had failed, possibly extinct in Slovenia (Potočnik, 1996). Despite this, it was not assigned a red list status in the national Red list of Malacostraca (Ur. l. RS., 2002).

The first recent observation was of a single isopod under a wooden board at the saltpans at Fontanigge in September 2023 (Figure 3A). Two days later, more than ten individuals were observed near the Walls of Piran, again under a wooden board. Six isopods found there were collected and preserved for further examination (Figure 3B). A third observation occurred at Fontanigge in March 2024. This time, more than ten individuals were observed under wooden pallets near the Museum of Salt Making (Figure 3C).

Species of the genus *Armadillo* are capable of rolling into a ball, with the flat protopodites of the uropods completing the outline of the pleon, and the uropod exopodites reduced to small cones that insert dorsally (Figure 3B). The genus is further characterized by monospiracular lungs on all pleopod exopodites and by modifications of pereopods 4 and 5 that enable these isopods to stridulate (Schmalfuss, 1996; Taiti et al., 1998; Cividini et al., 2020). While several species of *Armadillo* have been described from the Eastern Mediterranean, North Africa, and the Iberian Peninsula, *A. officinalis* is the only widespread Mediterranean species (Schmalfuss, 2003). It can be distinguished on the basis of the shape of the uropod protopodites and the pleotelson, the smoothness of the tergites, and male characters, mainly the shape of pereopod 7 and the exopodite of pleopod 1 (Schmalfuss, 1996).

The consistent presence of these isopods suggests that the population of *A. officinalis* in Slovenia may be recovering, with climate change possibly contributing to its success. Sket (2003) considered the species to be expanding its range, aided by human activity. Nevertheless, its recorded distribution in the country remains limited to the immediate proximity of the coast.

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Figure 3. *Armadillo officinalis*. A: The individual observed at Fontanigge in September 2023. B: One of the specimens collected in Piran in September 2023. The uropod protopodites complete the outline of the pleon and the uropod exopodites are diminutive (arrowhead). C: Individuals photographed at Fontanigge in March 2024. (photos A, B, C: Miloš Vittori).

Plecotus macrobullaris Kuzyakin, 1965 (Chiroptera, Mammalia) (bats, mammals)

Author(s)	Dren Dolničar
Corresponding author	Dren Dolničar (dren.dolnicar@gmail.com)
Leg.	Dren Dolničar
Country	Slovenia
Statement of significance	The highest elevation of recorded roost site for this species in Slovenia.
Locality description	Triglav National Park, Komna plateau, 1515 m a.s.l.
Habitat	An alpine meadow below the tree line, with a roost site in a wooden barn.
Date of observation	2024-07-11
Geographical coordinates	N 46.287972°, E 13.763278°
Voucher	/

On 11 July 2024, I found 2 individuals of *Plecotus* in the space between the planks and beams of the barn (WGS 84: 46.287972° N, 13.763278° E; Figure 4) next to the mountain hut Koča pod Bogatinom (address: Ukanc 148), on the Komna plateau, at 1515 m a.s.l. One was too far away to determine the species, while the other one, which was closer, I was able to identify as *Plecotus macrobullaris* based on the characteristic inverted triangular shape of the lower lip (Dietz & Kiefer, 2016). The bat, identified as *P. macrobullaris*, was initially positioned much closer to the edge of the gap between the barn's planks (Figure 4B), with its dark greyish triangular lower lip (Dietz & Kiefer, 2016) clearly visible. However, it retreated deeper into the gap after we disturbed it with light while attempting to photograph it.

The record is the highest roost of this species in Slovenia, since the previously highest elevation known roost was in the church sv. Duha in Podolševa at 1250 m a.s.l. (Presetnik et al., 2023). It is the third highest observation overall, as the highest published record originates from the mist netting at Planina pri Jezeru at 1750 m a.s.l. (Alberdi et al., 2013; CKFF, 2024) and the second highest is an ultrasound recording from Planina Krstenica at 1670 m a.s.l. (A. Zamolo, personal communication, August 28, 2024). According to the general species vertical distribution, the finding of *P. macrobularis* at this altitude on Komna is not surprising. The species is common in the Alpine regions and is found in all the main mountain ranges of the western Palearctic, stretching from the Pyrenees to the Middle East, and is distributed at altitudes ranging from sea level to 2800 m a.s.l. (Alberdi et al., 2013). The scarcity of observations of *P. macrobullaris* in Slovene montane belt (CKFF, 2024) therefore indicates a lack of research in the Slovenian high mountains.

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I would like to thank Primož Presetnik for gathering and sharing the relevant data about *P. macrobullaris* in Slovenia and for reviewing the first draft of this article and Aja Zamolo for sharing her records of this species. I would also like to thank Simona Strgulc Krajšek for sharing her photograph of *P. macrobullaris* that she took when I found it in the barn.

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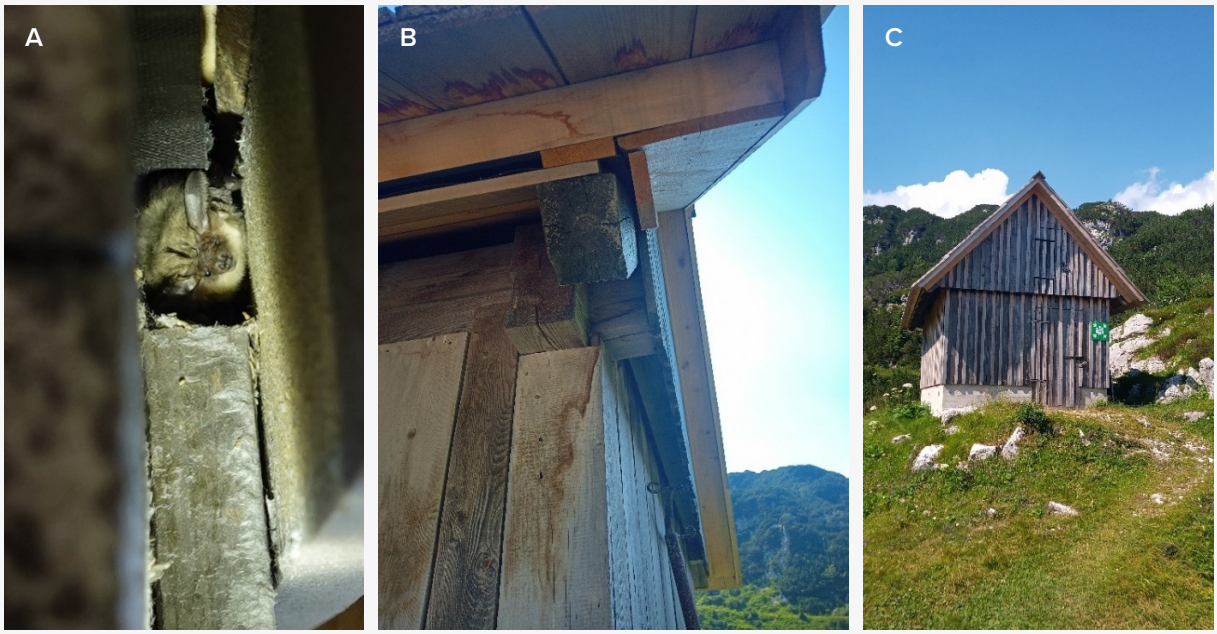

Figure 2. A) *Plecotus macrobullaris*; B) the roosting place; C) the barn near the hut Koča pod Bogatinom on Komna, where at least two *Plecotus* bats were roosting (photo: A: Simona Strgulc Krajšek; B, C: Dren Dolničar).

References

Alberdi, A., Garin, I., Aizpurua, O., Aihartza, J., 2013. Review on the Geographic and Elevational Distribution of the Mountain Long-Eared Bat *Plecotus macrobullaris*, Completed by Utilising a Specific Mist-Netting Technique. Acta Chiropterologica 15(2): 451–461. https://doi.org/10.3161/150811013X679071.

Blockeel, T. L., Bosanquet, S. D. S., Hill, M. O., Preston, C. D. (eds.), 2014. Atlas of British and Irish bryophytes: the distribution and habitat of mosses and liverworts in Britain and Ireland. Pisces Publications on behalf of the British Bryological Society.

Casas, C., Brugués, M., Cros, R. M., & Sérgio, C., 2006. Handbook of Mosses of the Iberian Peninsula. p. 311.

Cividini, S., Sfenthourakis, S., Montesanto, G., 2020. Are terrestrial isopods able to use stridulation and vibrational communication as forms of intra and interspecific signaling and defense strategies as insects do? A preliminary study in *Armadillo officinalis*. The Science of Nature, 107(1), 4. https://doi.org/10.1007/s00114-019-1656-3.

CKFF, 2024. *Plecotus macrobullaris*. Database of Center za kartografijo favne in flore, Miklavž na Dravskem polju. (July 2024, prepared by Primož Presetnik).

Dietz, C., Kiefer, A., 2016. Bats of Britain and Europe. Bloomsbury USA.

Ellis, L. T., Afonina, O. M., Czernyadjeva, I. V., Alegro, A., Šegota, V., Boiko, M., Zagorodniuk, N., Burghardt, M., Alataş, M., Aslan, G., Batan, N., Dragićević, S., Erata, H., Kırmacı, M., Özenoğlu, H., Evangelista, M., Valente, E. B., Feletti, T. A., Ezer, T., Fedosov, V. E., Fuertes, E., Oliván, G., Natcheva, R., Gospodinov, G., Hodgson, A., Kiebacher, T., Köckinger, H., von Konrat, M., Strgulc Krajšek, S., L. Cimerman, Ž., Kučera, J., Mikuláśková, E., Müller, F., Muñoz, J., Ochyra, R., Peralta, D. F., Philippe, M., Porley, R. D., Rawat, K. K., Paul, R. R., Ros, R. Werner, M., O., Schäfer-Verwimp, A., Sérgio, C., Shkurko, A. V., Söderström, L., de Souza, A. M., Spitale, D., Ştefănuţ, S., Tabua, M., Winter, G., 2022. New national and regional bryophyte records, 69, Journal of Bryology, 44:1, 87–102. https://doi.org/10.1080/03736687.2022.2061242.

Frey, W., Frahm, J. P., Fischer, E., Lobin, W., Blockeel, T. L. (eds.), 2006. The Liverworts, Mosses and Ferns of Europe. Harley Books, Essex, 512 pp.

Hill, M. O., Preston, C. D., 1998, The geographical relationships of British and Irish bryophytes, Journal of Bryology, 20:1, 127–226, https://doi.org/10.1179/jbr.1998.20.1.127.

Hill, M. O., Preston, C. D., Bosanquet, S. D. S., & Roy, D. B., 2007. BRYOATT : attributes of British and Irish mosses, liverworts and hornworts. Centre for Ecology and Hydrology.

Hodgetts, N. G., Lockhart, N., 2020. Checklist and country status of European bryophytes – update 2020. – Irish Wildlife Manuals 123. – Dublin: Department of Culture, Heritage and the Gaeltacht, 224 pp.

Hodgetts, N. G., Söderström, L., Blockeel, T. L., Caspari, S., Ignatov, M. S., Konstantinova, N. A., Lockhart, N., Papp, B., Schröck, C., Sim-Sim, M., Bell, D., Bell, N. E., Blom, H. H., Bruggeman-Nannenga, M. A., Brugués, M., Enroth, J., Flatberg, K. I., Garilleti, R., Hedenäs, L., Holyoak, D. T., Hugonnot, V., Kariyawasam, I., Köckinger, H., Kučera, J., Lara, F., Porley, R. D., 2020. An annotated checklist of bryophytes of Europe, Macaronesia and Cyprus. Journal of Bryology 42: 1–116.

Hodgetts, N., Cálix, M., Englefield, E., Fettes, N., García Criado, M., Patin, L., Nieto, A., Bergamini, A., Bisang, I., Baisheva, E., Campisi, P., Cogoni, A., Hallingbäck, T., Konstantinova, N., Lockhart, N., Sabovljević, M., Schnyder, N., Schröck, C., Sérgio, C., Sim Sim, M., Vrba, J., Ferreira, C. C., Afonina, O., Blockeel, T., Blom, H., Caspari, S., Gabriel, R., Garcia, C., Garilleti, R., González Mancebo, J., Goldberg, I., Hedenäs, L., Holyoak, D., Hugonnot, V., Huttunen, S., Ignatov, M., Ignatova, E., Infante, M., Juutinen, R., Kiebacher, T., Köckinger, H., Kučera, J., Lönnell, N., Lüth, M., Martins, A., Maslovsky, O., Papp, B., Porley, R., Rothero, G., Söderström, L., Ştefănuţ, S., Syrjänen, K., Untereiner, A., Váňa, J., Vanderpoorten, A., Vellak, K., Aleffi, M., Bates, J., Bell, N., Brugués, M., Cronberg, N., Denyer, J., Duckett, J., During, H. J., Enroth, J., Fedosov, V., Flatberg, K. I., Ganeva, A., Gorski, P., Gunnarsson, U., Hassel, K., Hespanhol, H., Hill, M., Hodd, R., Hylander, K., Ingerpuu, N., Laaka-Lindberg, S., Lara, F., Mazimpaka, V., Mežaka, A., Müller, F., Orgaz, J. D., Patino, J., Pilkington, S., Puche, F., Ros, R. M., Rumsey, F., Segarra-Moragues, J. G., Seneca, A., Stebel, A., Virtanen, R., Weibull, H., Wilbraham, J., Żarnowiec, J., 2019. A miniature world in decline: European Red List of Mosses, Liverworts and Hornworts. IUCN, Brussels. https://doi.org/10.2305/IUCN.CH.2019.ERL.2.en.

Lobnik Cimerman, Ž, Kopitar, D, Strgulc Krajšek, S., 2023. Arable bryophytes from Northeastern Slovenia with new and interesting national records. Botanica Serbica 47(2): 301–308. https://doi.org/10.2298/BOTSERB2302301C.

Long, D., 2010. *Riccia cavernosa*, Cavernous Crystalwort. In: Atherton, I., Bosanquet, S., Lewley, M. (eds.), Mosses and Liverworts of Britain and Ireland, a field guide. British Bryological Society, p 263.

Martinčič, A., 2016. Updated Red List of bryophytes of Slovenia. Hacquetia, 15(1), 107–126. https://doi.org/10.1515/hacq-2016-0006.

Martinčič, A., 2024. New checklist and the Red list of the hornworts (Anthocerotophyta) and liverworts (Marchantiophyta) of Slovenia. Hacquetia 23 (2): 175–197. https://doi.org/10.2478/hacq-2023-0013.

Martinčič, A., 2024. New Checklist and the Red list of the mosses (Bryophyta) of Slovenia. Hacquetia, 23(1), 69–118. https://doi.org/10.2478/hacq-2023-0006.

Müller, J., 2016. Einwanderung von *Cryphaea heteromalla* (Hedw.) D. Mohr nach Berlin und Brandenburg. Veröffentlichungen Des Naturkundemuseums Potsdam 2: 37–43.

Paton, A. J., 1999. The liverwort flora of the British Isles. Colchester: Harley Books.

Potočnik, F., 1984. Mokrice (Isopoda terrestria) slovenske obale Jadrana. Biološki vestnik, 32(1), 77–86.

Potočnik, F., 1989. The survey of the fauna of woodlices (Isopoda terrestria) of Yugoslavia. Biološki vestnik, 37, 61–82.

Potočnik, F., 1992. The red list of endangered Isopoda terrestria (Crustacea) in Slovenia. Varstvo narave, 17, 157–164.

Potočnik, F., 1996. Mokrice (Isopoda Terrestria). In: Gregori J, Martinčič A, Tarman K, Urbanc-Berčič O, Tome D, Zupančič M (eds.): Narava Slovenije, stanje in perspektive. Društvo ekologov Slovenije, Ljubljana, pp. 247–251.

Presetnik, P., Zamolo, A., Grgurevič, S., Šalamun, A., 2023. Monitoring populacij izbranih ciljnih vrst netopirjev v letih 2021–2023. Končno poročilo. Center za kartografijo favne in flore, Miklavž na Dravskem polju. 212 str., digitalne priloge. [Naročnik: Ministrstvo za naravne vire in prostor, Ljubljana].

Preston, C., 2010. *Cryphaea heteromalla*, Lateral Cryphaea. In: Atherton, I., Bosanquet, S., Lewley, M. (eds.), Mosses and Liverworts of Britain and Ireland, a field guide. British Bryological Society, p 672.

Schmalfuss, H., 1996. The terrestrial isopod genus *Armadillo* in Western Asia (Oniscidea: Armadillidae), with descriptions of five new species. Stuttgarter Beiträge zur Naturkunde, Serie A, 544.

Schmalfuss, H., 2003. World catalog of terrestrial isopods (Isopoda: Oniscidea). Stuttgarter Beiträge zur Naturkunde, Serie A, 654.

Sket, B., 2003. Raki – Crustacea. In: Sket B, Gogala M, Kuštor V (eds.): Živalstvo Slovenije. Tehniška založba Slovenije, Ljubljana, pp. 188–224.

Šraj-Kržič, N., Germ, M., Urbanc-Berčič, O., Kuhar, U., Janauer, G. A., Gaberščik, A., 2007. The Quality of the Aquatic Environment and Macrophytes of Karstic Watercourses. Plant Ecology 192(1): 107–118.

Strgulc Krajšek, S., Gey, S., Cezanne, R., Eichler, M., 2024. Report on the annual excursion of BLAM to Slovenia from September 13th to 17th, 2023. Herzogiella 11: 13–26.

Strouhal, H., 1929. Die Landisopoden des Balkans. 3. Beitrag: Südbalkan. Zeitschrift für wissenschaftliche Zoologie, 133, 57–120.

Taiti, S., Paoli, P., Ferrara, F., 1998. Morphology, biogeography, and ecology of the family Armadillidae (Crustacea, Oniscidea). Israel Journal of Zoology, 44, 291–301.

Ur. l. RS., 2002. Pravilnik o uvrstitvi ogroženih rastlinskih in živalskih vrst v rdeči seznam. Uradni list RS, no. 82/02, 42/10.

Vittori, M., Mavrič, B., Pekolj, A., Govedič, M., Zagmajster, M., 2023. A checklist of isopods (Crustacea: Isopoda) in Slovenia. Natura Sloveniae, 25(2), 27–52. https://doi.org/10.14720/ns.25.2.27-52.

ABS

Original Research

Cellulase, xylanase, lipase, and protease activities of selected wild mushrooms from Slovenia

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Abstract

There are more than 3000 species of wild mushrooms in Slovenia. These wild mushrooms represent a promising source of bioactive compounds that can also be used as a source of novel enzymes, thus offering potential economic benefits for a wide range of industries and medicine. In this work, the cellulase, xylanase, protease, and lipase activities were measured in aqueous extracts of 64 Slovenian wild mushrooms of different lifestyles (symbiotic, saprobic, parasitic). We have shown that many of the mushroom extracts tested contain a variety of specific hydrolases. Of the 64 wild mushroom extracts, 16 showed cellulase, 24 xylanase, 30 lipase, and five protease activities. These are the first reports of these enzymatic activities for some of the mushrooms tested.

Keywords

mushroom; enzyme; cellulase; xylanase; lipase; protease

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Celulazne, ksilanazne, lipazne in proteazne aktivnosti nekaterih samoniklih slovenskih gob

Izvleček

V Sloveniji je opisanih več kot 3000 gob, ki predstavljajo potencialni vir bioaktivnih molekul kot so biotehnološko uporabni encimi, ki bi se jih lahko uporabilo v različnih industrijskih procesih ali v medicini. V sklopu raziskave smo pripravili vodne izvlečke 64 različnih slovenskih samoniklih gob, ki smo jim določili celulazno, ksilanazno, proteazno in lipazno aktivnost. Pokazali smo, da ti vodni izvlečki vsebujejo različne kombinacije encimskih aktivnosti. V vodnih izvlečkih teh 64 različnih slovenskih gob smo pokazali, da ima 16 izvlečkov celulazno, 24 ksilanazno, 30 lipazno in pet proteazno aktivnost. Mnoge od teh aktivnosti v literaturi še niso bile opisane.

Ključne besede

gobe, encim, celulaze, ksilanaze, lipaze, proteaze

Introduction

The global market for industrial enzymes was estimated at USD 60.5 billion in 2023 and is expected to grow by 4.9 % annually from 2024 to 2030 (Enzymes Market Size, Share, Trends and Growth Report, 2030). Approximately 60 % of industrial enzymes are produced by fungi (Raveendran et al., 2018). Many of these enzymes are used in various industries, e.g. in the food, detergent, paper, and textile industries, as well as in medicine (Pandey et al., 2000). Proteases, which account for 60 % of industrial enzymes, catalyse the depolymerization of proteins to amino acids and are used in the detergent, pharmaceutical, and food industries (Singh et al., 2016; Raveendran et al., 2018). Lipases are enzymes that hydrolyse triglycerides into fatty acids and glycerol. They are indispensable for the production of food, biofuels, detergents, and animal feed (Raveendran et al., 2018). Cellulases are a group of hydrolases that act on the β-1,4-glycoside bond in cellulose to release glucose units (Raveendran et al., 2018). This class of enzymes is widely used in the production of food as well as in the textile, paper, and detergent industries (Sukumaran et al., 2005). Xylanases are a group of enzymes that cleave xylenes, which are a major component of the plant cell wall and are mainly used in the food industry (Raveendran et al., 2018). Although cellulases and xylanases have no pharmacological potential, this is not the case for lipases and proteases. Lipases are being actively investigated for the treatment of modern diseases such as obesity or even cancer (Jawed et al., 2019). Proteases, on the other hand, can be used to combat cardiovascular diseases, digestive disorders, and inflammatory diseases and to promote tissue repair in burns, bone fractures, or surgical trauma (Solanki et al., 2021).

Basidiomycota and Ascomycota are the major phyla of the fungal kingdom, and many of them produce spore-bearing fruiting bodies known as mushrooms (Schmidt-Dannert, 2016). As mushrooms can grow on a variety of substrates and have versatile lifestyles (symbiotic, saprobic, parasitic), they have evolved a powerful enzymatic mechanism. In this regard, mushrooms represent a promising source of novel and undiscovered enzymes that could offer great economic benefits to the industry. We, therefore, collected 65 wild mushrooms and determined their cellulase, xylanase, protease, and lipase activities.

Materials and Methods

Mushroom extract preparation

The mushrooms were collected in Slovenia (Table 1) between December 2019 and February 2021, identified to species level, cut into small pieces and frozen at -80 °C. To obtain aqueous mushroom extracts, the thawed fruiting bodies were homogenized in 50 mM TRIS-HCl buffer, pH 7.4. For 1 g of wet biomass, 1 mL of the pre-cooled buffer was used, except for mushrooms growing on wood, which absorbed more buffer. We mixed 2 mL of the pre-cooled buffer per 1 g of such wet mushrooms. Pieces of the thawed mushroom fruiting bodies were then homogenized in the

Waring Blender (Ika, Germany) for 10 seconds at maximal speed. Mushroom samples weighing less than 1 g were first frozen with liquid N2 and then homogenized in a mortar. Fruiting bodies that were homogenized using either a blender or a mortar were additionally homogenized using the Potter-Elvehjem homogenizer (Ika, Germany). Ten passages at 180 rpm were required to ensure complete homogenization of the mushroom biomass. The homogenized fungal biomass was then centrifuged at 4 °C and 11,900 × g for 30 min. 1 mL of the clear supernatant was then pipetted into a 1.5 mL centrifuge tube and frozen at -20 °C.

Commercial kits

The BCA assay (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify total proteins, the Megazyme endocellulase assay and the Megazyme endoxylanase assay (Megazyme, Bray, Ireland) were used to measure cellulase and xylanase activities, and the Protease Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure total protease activity. Cellulase, xylanase, and protease activities were determined by the protein concentration in the sample, which was the enzyme unit (EU)/mg protein.

Lipase activity

To determine the lipase activity, 50 mM 4-nitrophenyl butyrate (p-NPB) was prepared in acetonitrile as a substrate. After cleavage of the ester bonds in the substrate, a yellow product 4-nitrophenol is formed, which absorbs at 450 nm. For the lipase reaction, a lipase buffer containing 1 ml Triton X-100, 100 mM sodium phosphate and 150 mM NaCl, pH 7.2, was prepared. The aqueous mushroom extracts were thawed and incubated on ice before the experiment. First, 5 µl p-NPB and 450 µl lipase buffer were mixed and incubated at 37 °C for 10 min. Aqueous mushroom extract (45 µl) was then added, and after 10 min of incubation at 37 °C, the reaction was stopped by adding acetone (750 µl). The Eppendorf tubes were then centrifuged for 5 min at room temperature at 15,000 × g, and 200 µl of the supernatant was pipetted into the wells of a 96-well microtiter plate. For the negative control, 5 µl of lipase buffer was added instead of 5 µl of p-NPB. An additional negative control, which was prepared due to the strong absorption of p-NPB, consisted of 495 µl lipase buffer and 5 µl p-NPB. After incubation of negative control samples at 37 °C for 10 min, 750 µl acetone was added, and the mixture was centrifuged at room temperature for 5 min at 15,000 × g. 200 µl of the supernatant was pipetted into microtiter plates. The absorbance was measured using the VIS microplate reader (Dynex Technologies, Chantilly, VA, USA) at a wavelength of 450 nm. The test was repeated three times for each positive sample. Lipase activity was determined by the protein concentration in the sample as enzyme unit (EU)/mg protein. An EU is defined as the amount of sample that causes a change in absorbance at 450 nm of 1 mOD in one minute.

Table 1. Wild mushrooms were collected within this study.

Tabela 1. Divje gobe, zbrane v tej študiji.

Order	Family	Species	Bionomial name	Lifestyle[1]	Edibility[2]
Agaricales	Hygrophoraceae	*Hygrocybe conica*	*Hygrocybe conica* (Schaeff.) P. Kumm. 1871	a*	f
		Hygrocybe coccinea	*Hygrocybe coccinea* (Schaeff.) P. Kumm. 1871	a*	f
		Hygrocybe ovina	*Neohygrocybe ovina* (Bull.) Herink 1958	a*	d
		Hygrocybe citrinovirens	*Hygrocybe citrinovirens* (J.E. Lange) Jul. Schäff. 1947	a*	f
		Cuphophyllus pratensis	*Cuphophyllus pratensis* (Pers.) Bon 1985	a*	e
		Cuphophyllus fornicatus	*Cuphophyllus fornicatus* (Fr.) Lodge, Padamsee & Vizzini 2013	a*	f
		Gliophorus laetus	*Gliophorus laetus* (Pers.) Herink 1958	a*	f
		Porpolomopsis calyptriformis	*Porpolomopsis calyptriformis* (Berk.) Bresinsky 2008	a*	e

	Tricholomataceae	*Tricholoma stiparophyllum*	*Tricholoma stiparophyllum* (N. Lund) P. Karst. 1879	b	f
		Lepista nuda	*Lepista nuda* (Bull.) Cooke 1871	c	g
	Physalacriaceae	*Flammulina velutipes*	*Flammulina velutipes* (Curtis) Singer 1951	c	e
	Pluteaceae	*Volvopluteus gloiocephalus*	*Volvopluteus gloiocephalus* (DC.) Vizzini, Contu & Justo 2011	c	e
	Pleurotaceae	*Pleurotus ostreatus*	*Pleurotus ostreatus* (Jacq.) P. Kumm. 1871	a, c	e
		Pleurotus pulmonarius	*Pleurotus pulmonarius* (Fr.) Quél. 1872	a, c	e
	Schizophyllaceae	*Schizophyllum commune*	*Schizophyllum commune* Fr. 1815	a, c	f
	Marasmiaceae	*Megacollybia platyphylla*	*Megacollybia platyphylla* (Pers.) Kotl. & Pouzar 1972	c	e
	Amanitaceae	*Amanita eliae*	*Amanita eliae* Quél. 1872	b	d
		Amanita rubescens	*Amanita rubescens* Pers. 1797	b	g
		Amanita excelsa	*Amanita excelsa* (Fr.) Bertill. 1866	b	e
	Strophariaceae	*Stropharia eximia*	*Stropharia eximia* Benedix 1961	a	e
		Hypholoma fasciculare	*Hypholoma fasciculare* (Huds.) P. Kumm. 1871	a	d
	Omphalotaceae	*Gymnopus dryophilus*	*Gymnopus dryophilus* (Bull.) Murrill 1916	a	f
	Cortinariaceae	*Cortinarius bolaris*	*Cortinarius bolaris* (Pers.) Zawadzki 1835	b	f
	Nidulariaceae	*Cyathus striatus*	*Cyathus striatus* Willd. 1787	c	f
Russulales	Russulaceae	*Russula queletii*	*Russula queletii* Fr. 1872	b	f
		Russula virescens	*Russula virescens* (Schaeff.) Fr. 1836	b	e
		Russula nigricans	*Russula adusta* (Pers.) Fr. 1838	b	e
		Russula amoenolens	*Russula amoenolens* Romagn. 1952	b	f
		Russula cyanoxantha	*Russula cyanoxantha* (Schaeff.) Fr. 1863	b	e
		Lactarius torminosus	*Lactarius torminosus* (Schaeff.) Pers. 1797	b	f
		Lactarius volemus	*Lactifluus volemus* (Fr.) Kuntze 1891	b	e
		Lactarius vellereus	*Lactifluus vellereus* (Fr.) Kuntze 1891	b	f
Cantharellales	Hydnaceae	*Hydnum repandum*	*Hydnum repandum* L. 1753	b	e
	Cantharellaceae	*Cantharelus friesii*	*Cantharellus friesii* Quél. 1872	b	e
		Cantharellus cibarius	*Cantharellus cibarius* Fr. 1812	b	e
		Cantharellus amethysteus	*Cantharellus amethysteus* (Quél.) Sacc. 1887	b	e
Polyporales	Fomitopsidaceae	*Fomitopsis betulina*	*Fomitopsis betulina* (Bull.) B.K. Cui, M.L. Han & Y.C. Dai 2016	a, c	f

		Daedalea quercina	*Daedalea quercina* (L.) Pers. 1801	a, c	f
	Laetiporaceae	*Laetiporus sulfureus*	*Laetiporus sulphureus* (Bull.) Murrill 1920	a, c	g
	Polyporaceae	*Daedaleopsis confragosa*	*Daedaleopsis confragosa* (Bolton) J. Schröt. 1888	c	f
		Trametes hirsuta	*Trametes hirsuta* (Wulfen) Lloyd 1924	c	f
		Trametes versicolor	*Trametes versicolor* (L.) Lloyd 1921	c	f
		Trametes suaveolens	*Trametes suaveolens* (L.) Fr. 1838	c	f
		Trametes gibbosa	*Trametes gibbosa* (Pers.) Fr. 1838	c	f
		Fomes fomentarius	*Fomes fomentarius* (L.) Fr. 1849	a, c	f
		Neofavolus alveolaris	*Neofavolus alveolaris* (DC.) Sotome & T. Hatt. 2012	c	f
		Polyporus ciliatus	*Lentinus substrictus* (Bolton) Zmitr. & Kovalenko 2016	c	f
		Cerioporus leptocephalus	*Cerioporus leptocephalus* (Jacq.) Zmitr. 2016	c	f
	Meruliaceae	*Phlebia tremellosa*	*Phlebia tremellosa* (Schrad.) Nakasone & Burds. 1984	c	f
	Irpicaceae	*Gloeoporus taxicola*	*Meruliopsis taxicola* (Pers.) Bondartsev 1959	c	f
Auriculariales	Auriculariaceae	*Auricularia mesenterica*	*Auricularia mesenterica* (Dicks.) Pers. 1822	c	f
		Auricularia auricula-judae	*Auricularia auricula-judae* (Bull.) Quél. 1886	c	e
	Melanommataceae	*Phragmotrichum chailletii*	*Phragmotrichum chailletii* Kunze 1823	c	f
Hymenochaetales	Hymenochaetaceae	*Phylloporia ribis*	*Phylloporia ribis* (Schumach.) Ryvarden 1978	a, c	f
Boletales	Boletaceae	*Boletus reticulatus*	*Boletus reticulatus* Schaeff. 1774	b	e
		Strobilomyces strobilaceus	*Strobilomyces strobilaceus* (Scop.) Berk. 1851	b	f
		Tylopilus felleus	*Tylopilus felleus* (Bull.) P. Karst. 1881	b	f
	Tapinellaceae	*Tapinella atrotomentosa*	*Tapinella atrotomentosa* (Batsch) Šutara 1992	c	f
	Sclerodermataceae	*Scleroderma citrinum*	*Scleroderma citrinum* Pers. 1801	b	d
	Suillaceae	*Suillus luridiformis*	*Neoboletus luridiformis* (Rostk.) Gelardi, Simonini & Vizzini 2014	b	e
Gloeophyllales	Gloeophyllaceae	*Gloeophyllum odoratum*	*Gloeophyllum odoratum* (Wulfen) Imazeki 1943	c	f
Helotiales	Sclerotiniaceae	*Mitrula paludosa*	*Mitrula paludosa* Fr. 1816	c	f
	Chlorociboriaceae	*Chlorociboria aeruginascens*	*Chlorociboria aeruginascens* (Nyl.) Kanouse 1948	c	f
Pezizales	Tuberaceae	*Tuber aestivum*	*Tuber aestivum* (Wulfen) Spreng. 1827	b	e

[1] a*, parasitic biotrophs; a, parasitic; b, symbiotic; c, saprobic.

[2] d, poisonous; e, edible; f, inedible; g, conditionally edible.

Results

Of the 64 wild mushrooms examined, 23 were symbiotic fungi, and 41 had a saprobic or/and parasitic lifestyle. None of the examined mushrooms showed all four enzymatic activities, and 17 mushrooms had no enzymatic activities. In 23 mushroom samples, we could detect one or two enzymatic activities. The mushroom extracts from *Tricholoma stiparophyllum* and *Trametes* suaveolens showed three different enzymatic activities. We also investigated the correlations between the edibility (for humans) of the mushrooms and their enzymatic activities and found no correlations.

Cellulase activity

Sixteen of 64 wild mushrooms (25 %) showed cellulase activity (Figure 1), and 12 of these 16 mushrooms were not symbiotic. The highest cellulase activities were found in aqueous extracts of Fomitopsis betulina and Cyathus striatus. The mushrooms for which cellulase activity was already determined were *Pleurotus ostreatus, Pleurotus pulmonarius, Cyathus striatus, Fomitopsis betulina, Trametes versicolor, Trametes suaveolens, Trametes gibbosa* and *Auricularia auricula-judae* (Yerkes, 1967; Bhattacharjee et al., 1992; Valášková and Baldrian, 2006; Wang et al., 2014; Araújo et al., 2021; Beyisa Benti Diro et al., 2021; Montoya et al., 2021; Lu et al., 2022). Cellulase activity was determined for the first time for eight of these 16 wild mushrooms. These mushrooms were *Gliophorus laetus, Porpolomopsis calyptriformis, Tricholoma stiparophyllum, Volvopluteus gloiocephalus, Cantharelus friesii, Cantharellus amethysteus, Phragmotrichum chailletii* and *Suillus luridiformis*.

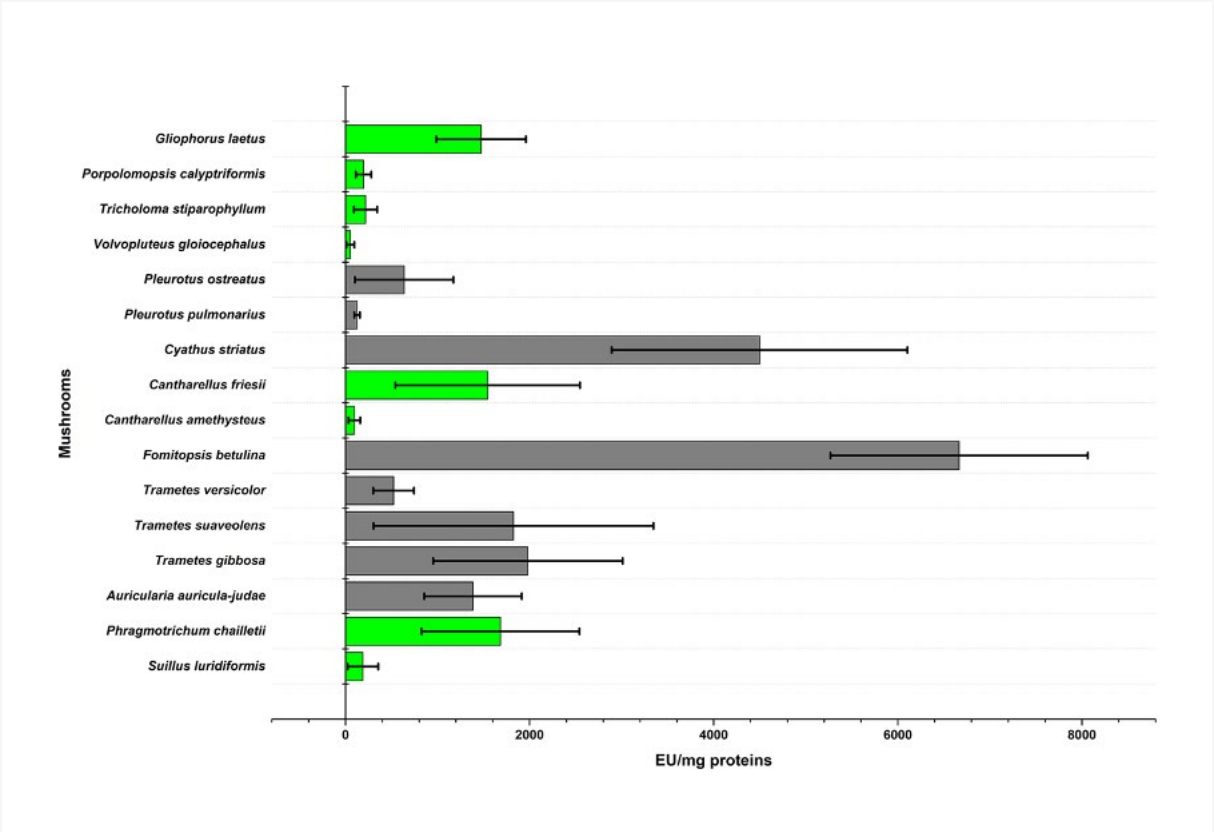

Figure 1. Aqueous extracts from wild mushrooms with detected cellulase activity. The mean values of triplicate samples with standard errors are shown. In green: the mushrooms for which cellulase activities are reported for the first time.

Slika 1. Vodni izvlečki divjih gob z ugotovljeno aktivnostjo celulaze. Prikazane so povprečne vrednosti trikratnih vzorcev s standardnimi napakami. V zeleni barvi: gobe, za katere so bile celulazne aktivnosti navedene prvič.

Xylanase activity

Twenty-four of 64 wild mushrooms (37.5 %) exhibited xylanase activity (Figure 2), of which 19 were non-symbiotic. The highest xylanase activities were found in aqueous extracts of *Phragmotrichum chailletii* and *Stropharia eximia*. The mushrooms for which xylanase activity has already been determined were *Pleurotus pulmonarius, Schizophyllum commune, Hydnum repandum, Fomitopsis betulina, Trametes versicolor* and *Trametes gibbosa* (Paice et al., 1978; Valášková and Baldrian, 2006; Inácio et al., 2015; Megersa and Alemu, 2019; Sergentani et al., 2016; Tišma et al., 2021). For 19 of these 24 wild mushrooms, we have demonstrated their xylanase activity for the first time. These mushrooms were *Hygrocybe ovina, Hygrocybe citrinovirens, Lepista nuda, Volvopluteus gloiocephalus, Amanita eliae, Stropharia eximia, Gymnopus dryophilus, Cyathus striatus, Russula nigricans, Cantharelus friesii, Trametes suaveolens, Neofavolus alveolaris, Polyporus ciliatus, Phlebia tremellosa, Gloeoporus taxicola, Phragmotrichum chailletii, Tylopilus felleus*, and *Chlorociboria aeruginascens*. The combined results of cellulase and xylanase activities of these wild mushrooms indicate that these two activities are more pronounced in non-symbiotic mushrooms.

Lipase activity

Our results show that of the four enzymatic activities tested, lipase activity is the most prevalent in the mushroom samples. Thirty of 64 wild mushrooms (46.9%) exhibited lipase activity (Figure 3), and it was somehow more pronounced in non-symbiotic mushrooms, as 19 of 30 mushrooms were non-symbiotic. *Flammulina velutipes, Daedaleopsis*

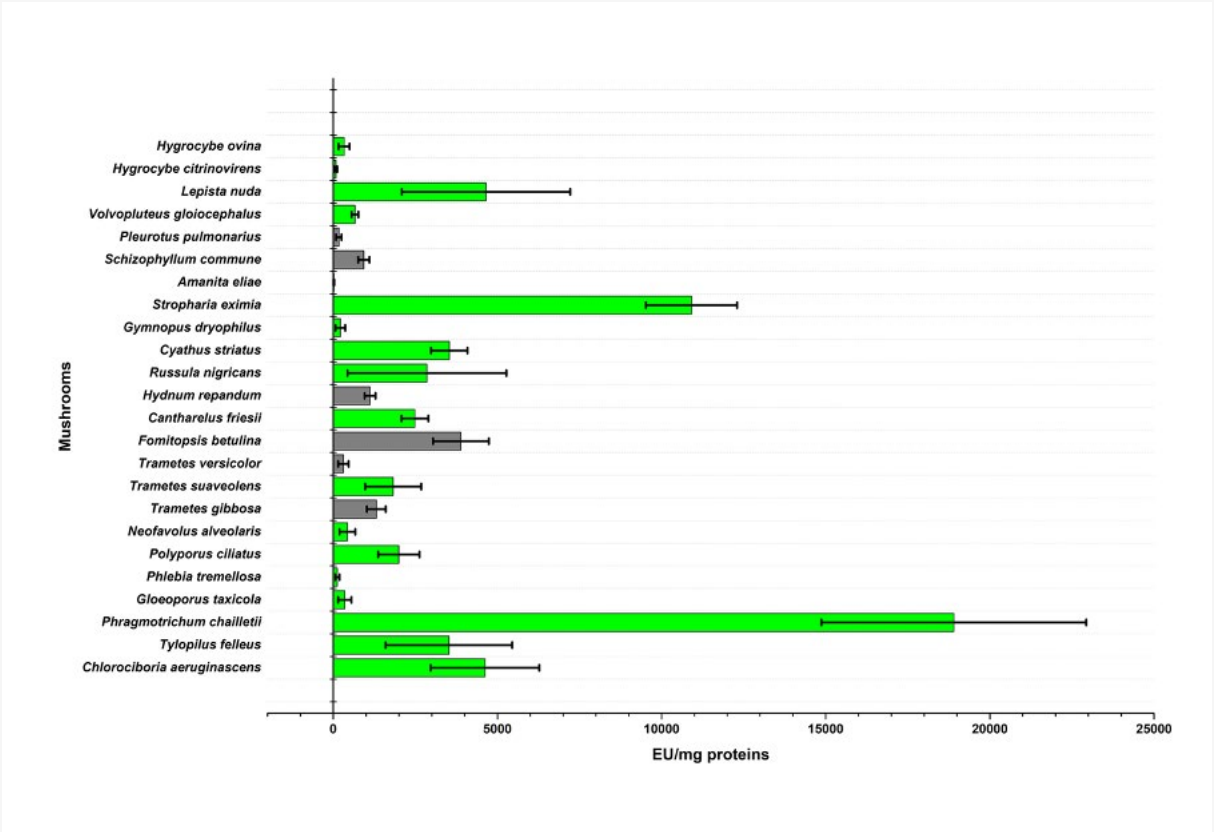

Figure 2. Aqueous extracts from wild mushrooms with detected xylanase activity. The mean values of triplicate samples with standard errors are shown. In green: the mushrooms for which xylanase activities were reported for the first time.

Slika 2. Vodni izvlečki divjih gob z ugotovljeno ksilanazno aktivnostjo. Prikazane so povprečne vrednosti trikratnih vzorcev s standardnimi napakami. V zeleni barvi: gobe, za katere so bile ksilanazne aktivnosti navedene prvič.

confragosa, Cuphophyllus fornicatus, Polyporus ciliates and *Lactarius torminosus* had the highest lipase activities. The mushrooms for which lipase activity had already been determined were *Schizophyllum commune, Amanita rubescens, Lactarius torminosus, Laetiporus sulfureus* and *Daedaleopsis confragosa* (Jaya et al., 2009; Krupodorova et al., 2014; Singh et al., 2015; Sepčić et al., 2019). We have demonstrated their lipase activity for 24 of these 30 wild mushrooms for the first time. These mushrooms were *Hygrocybe conica, Hygrocybe coccinea, Hygrocybe ovina, Cuphophyllus pratensis, Cuphophyllus fornicatus, Porpolomopsis calyptriformis, Tricholoma stiparophyllum, Lepista nuda, Flammulina velutipes, Megacollybia platyphylla, Amanita eliae, Stropharia eximia, Gymnopus dryophilus, Russula queletii, Russula virescens, Russula amoenolens, Lactarius volemus, Trametes suaveolens, Neofavolus alveolaris, Polyporus ciliatus, Phlebia tremellosa, Boletus reticulatus, Tylopilus felleus* and *Suillus luridiformis*. In addition, the five mushrooms that exhibited protease activity also showed lipase activity.

Protease activity

Five out of 64 wild mushrooms (7.8 %) showed protease activity (Figure 4). Four of the five mushrooms that showed protease activity were non-symbiotic fungi. The highest protease activity was found in the aqueous extract of *Daedaleopsis confragosa*. For all these five wild mushrooms, we are the first to detect their protease activity. These mushrooms were *Hygrocybe conica, Hygrocybe coccinea, Cuphophyllus pratensis, Tricholoma stiparophyllum* and *Daedaleopsis confragosa*.

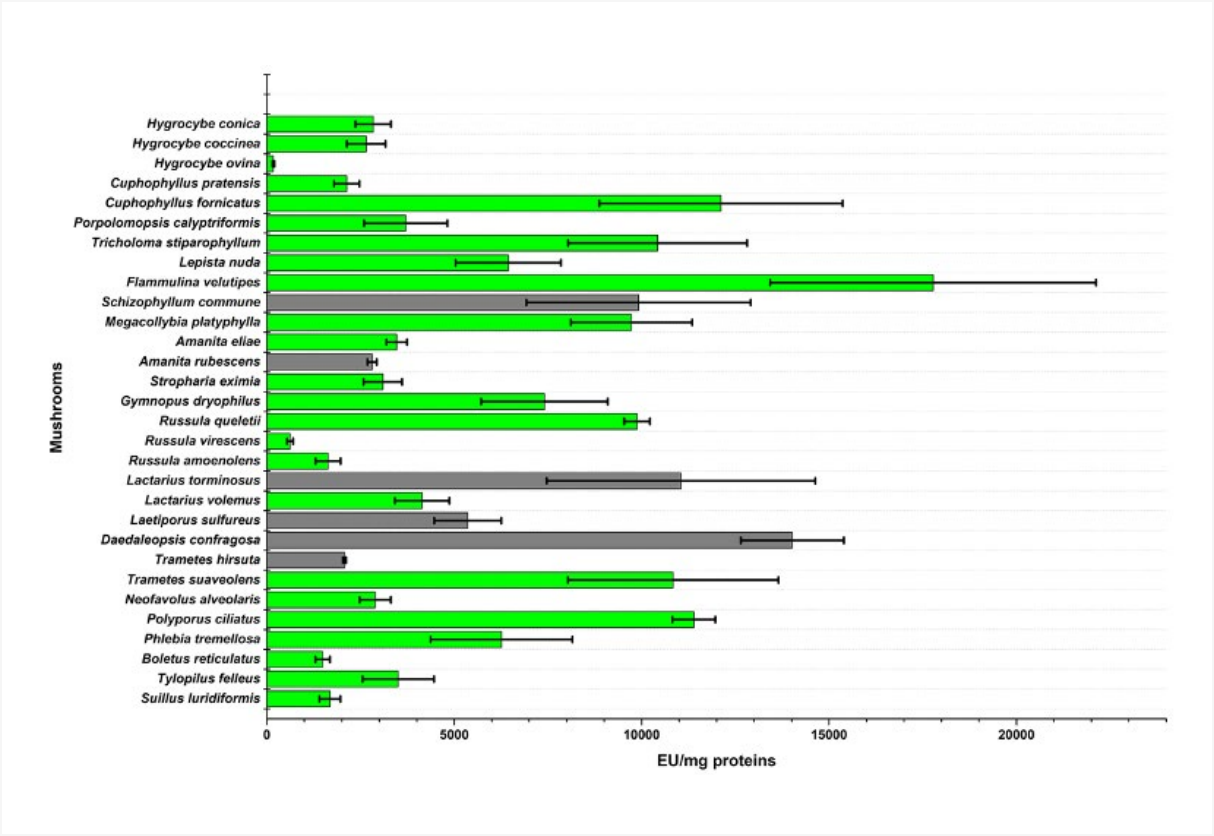

Figure 3. Aqueous extracts from wild mushrooms with detected lipase activity. The mean values of triplicate samples with standard errors are shown. In green: the mushrooms for which lipase activities are reported for the first time.

Slika 3. Vodni izvlečki divjih gob z ugotovljeno aktivnostjo lipaze. Prikazane so povprečne vrednosti trikratnih vzorcev s standardnimi napakami. V zeleni barvi: gobe, za katere so prvič poročali o aktivnosti lipaz.

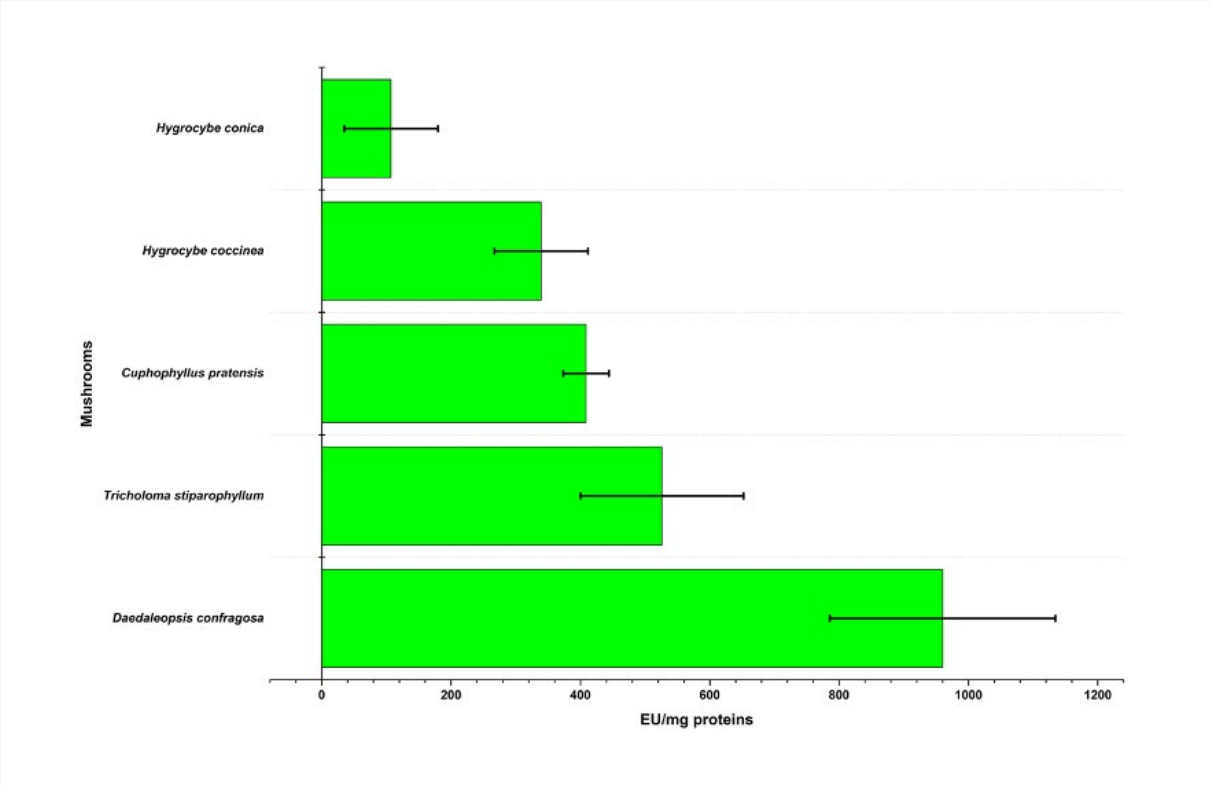

Figure 4. Aqueous extracts from wild mushrooms with detected protease activity. The mean values of triplicate samples with standard errors are shown. In green: the mushrooms for which protease activities are reported for the first time.

Slika 4. Vodni izvlečki divjih gob z ugotovljeno proteazno aktivnostjo. Prikazane so povprečne vrednosti trikratnih vzorcev s standardnimi napakami. V zeleni barvi: gobe, za katere so proteazne aktivnosti navedene prvič.

Discussion

Microbial hydrolases, including those from fungi, are the most important source of industrial enzymes. Fungal-derived enzymes account for more than 50% of the total enzyme market, and most of these enzymes are derived from moulds (El-Gendi et al., 2021). Almost 50% of these enzymes are produced by recombinant host strains, and most of them are also moulds (Arnau et al., 2019). Moulds are preferred enzyme factories because they can be grown in relatively inexpensive substrates under submerged conditions (Arnau et al., 2019). Since these enzymes are secreted extracellularly by moulds, they are relatively easy to separate from the liquid culture media. However, this does not apply to saprobic mushrooms, which have to be cultivated on solid culture media. The extraction of industrial enzymes from moulds is, therefore, less time-consuming, labour-intensive and more economically efficient than the extraction of enzymes from substrates overgrown with mushroom mycelia. While saprobic mushrooms can at least be cultivated on solid culture media, this is not always possible with symbiotic mushrooms, as only a few of them can be cultivated under laboratory conditions.

Alternative solutions for the production of mushroom enzymes should, therefore, be explored. One of the possible solutions is the cloning of genes from the producing mushrooms into suitable host(s). For this purpose, genome sequencing of mushrooms with high enzymatic activity should be performed. The target genes should be annotated, cloned and expressed in suitable microbial or fungal hosts, and the activity of these recombinant enzymes should be evaluated. By using recombinant technology to produce these enzymes in a host organism, it is possible to obtain large quantities of enzymes with the desired properties, which could lead to more efficient and sustainable industrial processes. In this context, Phragmotrichum chail-

letii and Stropharia eximia are proving to be a source of potent xylanases, Flammulina velutipes a source of potent lipase and Daedaleopsis confragosa a source of potent lipase and protease.

Although new fungal species have been identified in this work as producers of important industrial enzymes, it is not possible to compare these activities with the activities of pure enzymes derived from moulds grown under laboratory conditions. This is because these industrial mould strains grow in inducible substrates that trigger higher production of the enzymes. While the industrial enzymes produced by moulds are extracted directly from the liquid medium in which the moulds are grown, the extracts used in our study were derived directly from the producing organism. Since most symbiotic mushrooms cannot grow under laboratory conditions, recombinant expression of the target genes is the only solution for the evaluation and possible production of these enzymes on an industrial scale.

Conclusions

A growing number of industrial processes are replacing chemical-based solutions with biological-based solutions, which require new and effective enzymes. Our study, therefore, focused on the search for enzymes in higher fungi growing in nature, namely those that form fruiting bodies, which we believe represent an underestimated source of previously unexplored industrially important enzymes. The purpose of our study was to determine the cellulase, xylanase, lipase, and protease activities of 64 mushrooms collected in nature. This is the first report on the ability of many of these mushrooms to produce these hydrolytic enzymes. The findings of this study provide valuable insight into the occurrence of cellulases, xylanases, lipases, and proteases in wild mushrooms. They highlight, in particular, the diversity of mushrooms that can produce these enzymes. The use of recombinant technology to introduce genes encoding cellulase, xylanase, lipase and protease from mushrooms into a host organism may have various applications, particularly in industries such as biotechnology, agriculture, biofuel production and medicine.

Author Contributions

Conceptualization, M.S.; methodology, M.S. and K.S; validation, M.S.; formal analysis, I.P. and L.L.P; investigation, L.Š., L.L.P, I.P., M.H. and D.F.; resources, M.S. and K.S.; data curation, M.S.; writing—original draft preparation, M.S.; writing—review and editing, M.S., L.Š., I.P., M.H., D.F. and K.S.; visualization, L.P.P.; supervision, M.S.; project administration, M.S.; funding acquisition, M.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability

All raw or analysed data are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

References

Araújo, N.L., Avelino, K.V., Halabura, M.I.W., Marim, R.A., Kassem, A.S.S., Linde, G.A., ..., do Valle, J.S., 2021. Use of green light to improve the production of lignocellulose-decay enzymes by *Pleurotus* spp. in liquid cultivation. Enzyme and Microbial Technology, 149, 109860. https://doi.org/10.1016/j.enzmictec.2021.109860

Arnau, J., Yaver, D., Hjort, C.M., 2019. Strategies and Challenges for the Development of Industrial Enzymes Using Fungal Cell Factories. Grand Challenges in Fungal Biotechnology, 179–210. https://doi.org/10.1007/978-3-030-29541-7_7

Diro, B.B., Daba, T., Genemo, T.G., 2021. Production and characterization of cellulase from mushroom (*Pleurotus ostreatus*) for effective degradation of cellulose. International Journal of Biological and Pharmaceutical Sciences Archive, 2(1), 135–150. https://doi.org/10.53771/ijbpsa.2021.2.1.0066

Bhattacharjee, B., Roy, A., Majumder, A.L., 1992. Beta-glucosidase of a white-rot fungus *Trametes gibbosa*. Biochemistry International, 28(5), 783–793

El-Gendi, H., Saleh, A.K., Badierah, R., Redwan, E.M., El-Maradny, Y.A., El-Fakharany, E.M., 2021. A Comprehensive Insight into Fungal Enzymes: Structure, Classification, and Their Role in Mankind's Challenges. Journal of Fungi, 8(1), 23. https://doi.org/10.3390/jof8010023

Enzymes Market Size, Share, Trends and Growth Report, 2030. (n.d.). Retrieved January 31, 2024, from https://www.grandviewresearch.com/industry-analysis/enzymes-industry

Inácio, F.D., Ferreira, R.O., de Araujo, C.A.V., Peralta, R.M., de Souza, C.G.M., 2015. Production of Enzymes and Biotransformation of Orange Waste by Oyster Mushroom, *Pleurotus pulmonarius* (Fr.) Quél. Advances in Microbiology, 5(1), Article 1. https://doi.org/10.4236/aim.2015.51001

Jawed, A., Singh, G., Kohli, S., Sumera, A., Haque, S., Prasad, R., Paul, D., 2019. Therapeutic role of lipases and lipase inhibitors derived from natural resources for remedies against metabolic disorders and lifestyle diseases. South African Journal of Botany, 120, 25–32. https://doi.org/10.1016/j.sajb.2018.04.004

Jaya, M., Goud, P., Suryam, A., Vadlakonda, L., Charya, M., 2009. Extracellular hydrolytic enzyme profiles of certain South Indian basidiomycetes. African Journal of Biotechnology, 8

Krupodorova, T., Ivanova, T., Barshteyn, V., 2014. Screening of extracellular enzymatic activity of macrofungi. Journal of Microbiology, Biotechnology and Food Sciences, 3, 315–318

Lu, X., Li, F., Zhou, X., Hu, J., Liu, P., 2022. Biomass, lignocellulolytic enzyme production and lignocellulose degradation patterns by *Auricularia auricula* during solid state fermentation of corn stalk residues under different pretreatments. Food Chemistry, 384, 132622. https://doi.org/10.1016/j.foodchem.2022.132622

Megersa, S., Alemu, M., 2019. Qualitative assays and quantitative determinations of xylanolytic enzymes of wood rot fungi from Dagaga and Gambo forests, Ethiopia. Journal of Bioscience and Biotechnology Discovery, 4(4), 69-78. https://doi.org/10.31248/JBBD2019.101

Montoya, S., Patiño, A., Sánchez, Ó.J., 2021. Production of Lignocellulolytic Enzymes and Biomass of *Trametes versicolor* from Agro-Industrial Residues in a Novel Fixed-Bed Bioreactor with Natural Convection and Forced Aeration at Pilot Scale. Processes, 9(2), Article 2. https://doi.org/10.3390/pr9020397

Paice, M.G., Jurasek, L., Carpenter, M.R., Smillie, L.B., 1978. Production, characterization, and partial amino acid sequence of xylanase A from *Schizophyllum commune*. Applied and Environmental Microbiology, 36(6), 802–808. https://doi.org/10.1128/aem.36.6.802-808.1978

Pandey, A., Nigam, P., Soccol, C.R., Soccol, V.T., Singh, D., Mohan, R., 2000. Advances in microbial amylases. Biotechnology and Applied Biochemistry, 31(2), 135–152. https://doi.org/10.1042/ba19990073

Raveendran, S., Parameswaran, B., Ummalyma, S.B., Abraham, A., Mathew, A.K., Madhavan, A., Rebello, S., Pandey, A., 2018. Applications of Microbial Enzymes in Food Industry. Food Technology and Biotechnology, 56(1), 16–30. https://doi.org/10.17113/ftb.56.01.18.5491

Schmidt-Dannert, C. 2016. Biocatalytic Portfolio of Basidiomycota. Current Opinion in Chemical Biology, 31, 40–49. https://doi.org/10.1016/j.cbpa.2016.01.002

Sepčić, K., Sabotič, J., Ohm, R.A., Drobne, D., Jemec Kokalj, A., 2019. First evidence of cholinesterase-like activity in Basidiomycota. PLoS ONE, 14(4), e0216077. https://doi.org/10.1371/journal.pone.0216077

Sergentani, A.G., Gonou-Zagou, Z., Kapsanaki-Gotsi, E., Hatzinikolaou, D.G., 2016. Lignocellulose degradation potential of Basidiomycota from Thrace (NE Greece). International Biodeterioration & Biodegradation, 114, 268–277. https://doi.org/10.1016/j.ibiod.2016.07.004

Singh, J., Singh, M.K., Kumar, M., Thakur, I.S., 2015. Immobilized lipase from Schizophyllum commune ISTL04 for the production of fatty acids methyl esters from cyanobacterial oil. Bioresource Technology, 188, 214–218. https://doi.org/10.1016/j.biortech.2015.01.086

Singh, R., Mittal, A., Kumar, M., Mehta, P., 2016. Microbial Proteases in Commercial Applications. https://www.semanticscholar.org/paper/Microbial-Proteases-in-Commercial-Applications-Singh-Mittal/c35dbf37afd2bcda38101e5425b3009e39c4e174

Solanki, P., Putatunda, C., Kumar, A., Bhatia, R., Walia, A., 2021. Microbial proteases: Ubiquitous enzymes with innumerable uses. 3 Biotech, 11(10), 428. https://doi.org/10.1007/s13205-021-02928-z

Sukumaran, R.K., Singhania, R.R., Pandey, A., 2005. Microbial cellulases-Production, applications and challenges. Journal of Scinece & Industrial Research 64(11), 832-844

Tišma, M., Žnidaršič-Plazl, P., Šelo, G., Tolj, I., Šperanda, M., Bucić-Kojić, A., Planinić, M., 2021. *Trametes versicolor* in lignocellulose-based bioeconomy: State of the art, challenges and opportunities. Bioresource Technology, 330, 124997. https://doi.org/10.1016/j.biortech.2021.124997

Valášková, V., Baldrian, P., 2006. Degradation of cellulose and hemicelluloses by the brown rot fungus *Piptoporus betulinus*—Production of extracellular enzymes and characterization of the major cellulases. Microbiology (Reading, England), 152(Pt 12), 3613–3622. https://doi.org/10.1099/mic.0.29149-0

Wang, W., Li, Y., Wang, H., Zu, Y., 2014. Differences in the Activities of Eight Enzymes from Ten Soil Fungi and Their Possible Influences on the Surface Structure, Functional Groups, and Element Composition of Soil Colloids. PLOS ONE, 9(11), e111740. https://doi.org/10.1371/journal.pone.0111740

Yerkes, J.W.D., 1967. Process for preparing a cellulolytic enzyme from *Trametes* suaveolens (United States Patent US3310476A). https://patents.google.com/patent/US3310476A/en

Original Research

Phytochemical composition and antimicrobial activity of essential oil and extracts from the leaves of *Peganum harmala* L. in southern Algeria.

Ghillace Abderrahmane [1*], Chouitah Ourida [1], Kiari Fatima Zohra [1], Fergoug Zineb [2], Daikh Zineeddine [2]

Abstract

Medicinal plants have a wide use in traditional medicine. The aim of our study is tovalorize medicinal and aromatic plants of the Algerian flora. For this reason, we determined the phytochemical composition and antimicrobial activity of the essential oil of *Peganum harmala* in two different southern regions. Phytochemical screening reveals the presence of flavonoids, alkaloids, saponins, tannins, glycosides, terpenoids and steroids and the absence of anthraquinonesin extracts. The essential oil of *P.harmala* leaves collected from two different regions (Bougtob and Mechria)was analyzed by GC-MS. The results revealed the presence of 55 and 47 compounds with a yield of 0.217 % and 0.211 %. These essential oils do not contain the same major components for Bougtob region: Carvone 21.15%, Phytol 17.41%, Carbamic acid 3.52%, Lauric acid 2.10%, Thymol 1.77% and Tetrapentacontane 1.32%. For the region of Mechria, Phytol 10.24%, Carvone 5.92%, Tetrapentacontane 2.80%, Phytone 2.18%, Butylated Hydroxytoluene 1.48%, Lauric acid 1.39%. The essential oils which were tested showed variable degrees of antimicrobial activity. In general, Gram-positive bacteria and fungi were found to be more sensitive to the essential oils than Gram-negative bacteria.

Keywords

Peganum harmala, Leaves, Essential oil and extracts,GC-MS, Phytochemical screening, Antimicrobial activity, southern Algeria.

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Fitokemična sestava in protimikrobna aktivnost eteričnega olja in izvlečkov iz listov *Peganum harmala* L. v južni Alžiriji.

Izvleček

Zdravilne rastline imajo široko uporabo v tradicionalni medicini. Namen naše raziskave je valorizirati zdravilne in aromatične rastline alžirske flore, zato smo določili fitokemično sestavo in protimikrobno delovanje eteričnega olja *Peganum harmala* v dveh različnih južnih regijah. Fitokemijski pregled razkrije prisotnost flavonoidov, alkaloidov, saponinov, taninov, glikozidov, terpenoidov in steroidov ter odsotnost antrakinonov v izvlečkih. Eterično olje listov *P.harmala*, zbranih iz dveh različnih regij (Bougtob in Mechria), je bilo analizirano z GC-MS. Rezultati so pokazali prisotnost 55 in 47 spojin z izkoristkom 0,217 % in 0,211 %. Ta eterična olja ne vsebujejo istih glavnih sestavin za regijo Bougtob Carvone (21,15 %), fitol (17,41 %), karbamsko kislino (3,52 %), lavrinsko kislino (2,10 %), timol (1,77 %) in tetrapentakontan (1,32 %). Za regijo Mechria, fitol (10,24 %), karvon (5,92 %), tetrapentakontan (2,80 %), fiton (2,18 %), butil hidroksitoluen (1,48 %), lavrinska kislina (1,39 %). Eterična olja, ki so bila testirana, so pokazala različne stopnje protimikrobnega delovanja. Na splošno je bilo ugotovljeno, da so po Gramu pozitivne bakterije in glive bolj občutljive na eterična olja kot po Gramu negativne bakterije.

Ključne besede

Peganum harmala, listi, eterično olje in izvlečki, GC-MS, fitokemični pregled, protimikrobna aktivnost, južna Alžirija.

Introduction

Since the highest antiquity, Men have used plants found in nature in several fields, such as perfumery, pharmacology, agri-food, and disease treatments. (Roosta et al., 2017)

More than 20,000 aromatic and medicinal plants are used in traditional medicine (Pandey et al., 2013). These plants continue to provide valuable therapeutic agents for both modern and traditional medicine (Bouzouita et al., 2008).

Aromatic plants are characterized by the biosynthesis of odorous molecules, which constitute the essential oils known for their antiseptic and therapeutic activity in popular medicine for a long time (Samarth et al., 2017). Essential oils are complex natural substances with well-defined physicochemical characteristics and meet quality criteria that must be known to avoid any risk of toxicity, which may prove dangerous to healthbecausesome essential oils and medicinal plants are very dangerous (Haddouchi et al., 2008) in except to those of some medicinal plants. In addition, these products have a great variability in their chemical constituents, giving them many medicinal and biological properties that should be known and valued (Rashid et al., 2010).

The great use of antibiotics in medicine to fight pathogenic micro-organisms is not completely harmless to the body and can cause many undesirable effects (Rudramurthy et al., 2016). Itclearly drives the evolution of resistance (CDCPOIDA, 2013). Epidemiological studies have demonstrated a direct relationship between antibiotic consumption and the emergence and dissemination of resistant bacteria strains.In bacteria, genes can be inherited from relatives or can be acquired from nonrelatives on mobile genetic elements such as plasmids. This horizontal gene transfer (HGT) can allow antibiotic resistance to be transferred among different species of bacteria. Resistance can also occur spontaneously through mutation. Antibiotics remove drug-sensitive competitors, leaving resistant bacteria behind to reproduce as a result of natural selection (Read et al., 2014). Despite warnings regarding overuse, antibiotics are overprescribed worldwide (The Antibiotic Alarm, 2013).Therefore, it is necessary to search for new natural antimicrobial molecules to inhibit various pathogenic microorganisms (Rudramurthy et al., 2016).

Algeria, by its geographical location, offers rich and diverse vegetation. A large number of aromatic plants grow there spontaneously. Interest in these plants has continued to grow in recent years. The Algerian flora includes 3300

species (Quezel& Santa, 1963). *Peganum harmala* L., commonly known as Syrian rue or Pègano, is a herbaceous plant belonging to the Zygohpyllaceae family. The primary origin of *Peganum harmala* is central Asia, but nowadays, it grows in Australia, north of Africa and southwest of Amecira (Bahmani et al., 2012). It is an herbaceous plant, glabrous, 30 to 60 cm in height with a lignified base, much branched with a winged stem at the top and alternate leaves. It possesses narrow leaves arranged alternately on fleshy, bright green stiff stems (Zargari et al., 1992). The flowers of the plant are isolated in the axils of the leaves,1-2 cm, whitish-green in colour and round seed capsules containing more than 50 seeds. It has a strong, unpleasant odour (Zargari et al., 1992; Marwat et al., 2011).

Many related works show that *Peganum harmala* essential oils have antibacterial, antiviral, antioxidant, antiparasitic and anticancer activities, among other biological activities. The purpose of our research is toevaluate the chemical composition and antimicrobial activity of essential oils extracted from the leaves of *Peganum harmala*. This study aims to valorize medicinal and aromatic plants of the Algerian flora in order to find new bioactive natural products by the evaluation of the chemical composition and antimicrobial activity of essential oils which are extracted from the leaves of *P. harmala* collected from two different regions (Bougtob and Mechria).

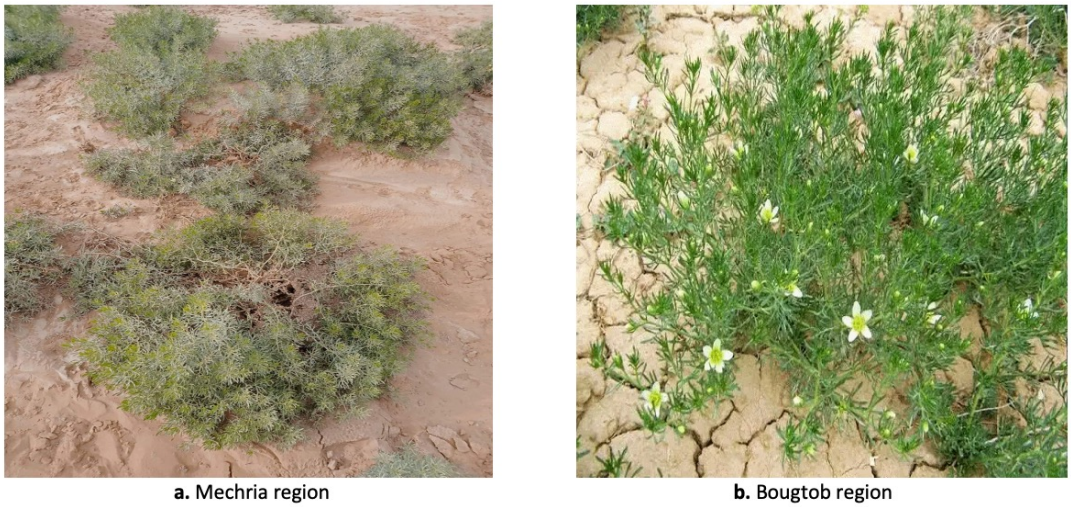
Materials and Methods

Plant material

The leaves of *P.harmala* (Figure 1) were collected in April 2020 from the Bougtob region (ElBayed-Algeria) and Mechria region (Naama-Algeria), situated in the South of Algeria. The plant was identified by a botanist from the Faculty of Science and Lifeat the University of Mascara. The leaves were cleaned, shaded, dried, and conserved in a closed and dark room.

Essential oil extraction

For the extraction of essential oils by hydrodistillation, Clevenger-type was used (Chouitah et al., 2017), and the dried plant 50g was submitted in a balloon of 500 ml for 3 hours. Sodium sulfate anhydrous(Na_2SO_4) (used to dry the extract) was added to the essential oil that was recovered and stored in the refrigerator at 4°C and in the dark until use.



a. Mechria region
a. regijo Mechria

b. Bougtob region
b. regijo Bougtob

Figure 1. *Peganum harmala* L. plant

Slika 1. Rastlina *Peganum harmala* L.

The yield of essential oil was determined by its contribution to the dry material.

$R (\%) = (m / m_0) \times 100$

Where m: essential oil mass (g), m_0: fresh leaves mass (g), R: essential oil yield (%) (Boutekedjiret et al., 2003).

Preparation of extracts

Ten grams of each powder sample (leaves) were quenched separately in 100 ml of hydromethanolic solution (water/ methanol 20:80 v/v) for 48 hours with stirring to obtain two extracts (Mechria and Bougtob region hydromethanolic extracts). The extracts were then filtered using a Whatman No. 1 filter paper. All filtrates were pressured to a boiling temperature of 50 °C, then they dried at 40 °C and stored at 4 °C until their analysis (Pharmacognosie, 1999).

Analysis of the essential oils

According to AFNOR NF ISO 280: 1999, essential oils must respond to analytical characteristics that are established by international committees of experts. Standard organoleptic tests and measuring of some physicochemical parameters were performed.

Organoleptic characters

It is a very important test in determining the quality of an essential oil. It is based on the colour, smell and appearance of the essential oil and is carried out according to AFNOR NF ISO 280 (1999).

Physical properties of the essential oil

Essential oil is said to be miscible with V volume and more ethanol of alkalimetric strength determined at a temperature of 20°C (AFNOR 75-101, 2000).When the mixture of 1 volume of the essential oil with V volumes of this ethanol is clear, and the remainder after gradual addition of ethanol of the same strength up to a total of 20 volumes.

Briefly, ethanol of suitable alcoholic strength is gradually added to a test portion of essential oil at a temperature of 20°C, and then their miscibility was evaluated by stirring vigorously during the addition of the solvent. When the obtained solution was perfectly clear, note that the volume (V) of dilution of ethanol was used with continuous addition of solvent in 0.5ml portions up to a total of 20ml, and stirring was performed after each addition.The miscibility of essential oils with ethanol of title t, at a temperature of 20°C, is expressed by One volume of essential oils in V volumes of ethanol with title t.

The acid index (NF T 75 103, 1982)expresses the number of milligrams of potassium hydroxide (KOH) required to neutralize the free acids contained in one gram of essential oil. 2 g of essential oil was added to 5 ml of ethanol 95% and five drops of phenolphthalein at 0.2%. The solution was neutralized by the solution of KOH (0.1 mol / l) until a pink colour. We denoted the volume of the KOH solution added. The calculation of AI is given by the formula: AI = 5.61 x V / M;5.61: Corresponds to 0.1 mol / L KOH; M: mass in grams of the essential oil; V: Volume in millilitres of ethanol solution of KOH (0.1 mol / l) used for titration.

The ester index value(NFT 75 104, 1982) is the number of milligrams of KOH needed to neutralize the free acids by hydrolysis of esters contained in one gram of essential oil. 2 g of essential oil was added to 25ml of ethanol solution of KOH (0.5 mol / l). It adopted the condenser, placed the ball on the heating mantle and allowed it to heat for one hour. Then, the solution was added to 20 ml of distilled water and five drops of 0.2% PP. The excess KOH solution was titrated with hydrochloric acid 0.5 mol / l. A blank test was carried out under the same conditions and with the same reagents. The calculation of EI is given by the formula: $EI = (28.05 \times (V_0-V_1) / M)-IA$; 28.05 g / l: corresponding to 0.5 mol / L KOH; M: mass in grams of the test; V_0: Volume in ml of the HCl solution (0.5 mol / l) used for the blank; V_1: volume in ml of the HCl solution (0.5 mol / l) used to determine the EI of the EO.

Physical properties of the essential oil

Relative density was performed using a pycnometer with a volume of 1 ml and temperature of 20 °C(NF T 75 111, 1982). Rotator power was obtained according to NF T 75 113 (1982)using a polarimeter-type VISTA C25. Light source (sodium-vapour lamp) for obtaining a light wavelength of 589.3 nm ± 0.3 and a viewing tube 100 ± 0.5 mm in length. The refractive index was carried out using a refract meter to Bellingham type with a precision of ± 0.0002, a direct reading of refractive index between 1.3000 and 1.7000 situated(NF T 75 112, 1977).The freezing point was determined according to AFNOR 75-111 (2000). The essential oil was put in test tubes inside a freezer, accompanied

by a thermometer, hence the observation of temperature variations accompanying the solidification of the oil, which was cooled slowly and gradually.

Phytochemical screening

Phytochemical analysis of plant extracts is necessary to reveal the presence of certain chemical families. The detection of different phytochemical components of *P.harmala* extracts was performed by chemical detection tests based on phenomena of precipitation or colouration using the methods described in the literature.To test for phenols, we added to 2 ml of each extract solution a drop of 2 % $FeCl_3$ alcoholic solution. The positive reaction was expressed by the appearance of a green or blue-black colouration (Adou et al., 2016). For the test of flavonoids, we added 0.5 ml of each extract, ten drops of concentrated hydrochloric acid and a few milligrams of sunflower magnesium. Positive results were indicated by a pink-red or yellow colouration after three minutes of incubation (Haddouchi et al., 2016). To test for tannins, we took 3 ml of extract, placed it in a test tube and diluted with chloroform. 1 ml of acetic anhydride was added. Then, 1 ml of sulphuric acid was poured in carefully. A green colour was obtained, indicating the presence of tannins (Hossain et al., 2013).For the test for saponins, the dry powder was vigorously mixed with water. A positive result was indicated by the appearance of foam for more than 5 minutes (Joshi et al., 2013).For the test for coumarin glycosides, a few drops of $FeCl_3$ alcoholic solution were added to each extract. The presence of coumarins is indicated by the appearance of a green colouration after the addition of concentrated HNO_3 (Joshi et al., 2013).For the test for anthraquinones, approximately 0.5 g of the extract was stirred with 5 ml chloroform for ten minutes and filtered. The filtrate was shaken with 5 ml of ammonia solution. The presence of pink colour in the ammonia phase indicates the presence of anthraquinones. For a combined anthraquinones test, 1 g of powdered extract boiled with 5 ml of 10 % hydrochloric acid for five minutes. The cold filtrate was diluted with chloroform. The chloroform layer was then transferred to a test tube. An equal volume of 10 % ammonia was added to the chloroform extract. The presence of combined anthraquinones was indicated by a pink, red, or purple colour (Abodunrin et al., 2015). For the test for alkaloids, 0.5 g of the extract was mixed with 5 ml of 1 % aqueous hydrochloric acid in a water bath. A few drops of Dragendorff's reagent were added to 1 ml of the filtrate. Turbidity or precipitation was considered to have a positive result (Ismail et al., 2016).For the test for steroids, 2 ml acetic anhydride and 2 ml H_2SO_4 were added to 5 ml of the extract. The colour change from violet to blue confirmed the presence of steroids (Kumar, 2015).For the test for proteins, 2 ml of NaOH (4 %) and a few drops of 3 % $CuSO_4$ solution were mixed with 2 ml of solution extract. The presence of protein is indicated by the formation of purple or pink colour (Maria et al., 2018).For the test of quinones, 3 ml of each extract was treated with 3 ml of chloroform, and then the chloroform layer was separated. A volume ofpotassium hydroxide (5 %) was then added to the chloroform layer. The appearance of red colour indicates the presence of quinones (Amezouar et al., 2013).

Chromatographic analysis: Gas chromatography/mass spectrometry analysis (GC-MS)

The volatile constituents of the essential oil were analyzed by "Shimadzu" GCMS-TQ8030 Gas chromatograph mass spectrometer with a Stabilwax silica capillary column (30 m X 0.32 mm, film thickness, 0.25 µm).Maximum Temp 350°C. The carrier gas was helium, Column Oven Temperature: 50°C, Injection Temperature: 220°C, rate changeof 5°C/ min, Equilibrium Time: 3min, vial capacity: 1.5 ml, and syringe capacity: 10µl.

Microbial strains

The antimicrobial activity of the essential oil samples was tested against seven different microorganisms: The strains of the international ATCC collection (American Type Culture Collection).

Gram+: *Staphylococcus aureus* ATCC 2592, *Bacillus cereus* ATCC 11778.

Gram- : *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700603, *Escherichia coli* ATCC 25922.

Fungus: *Candida albicans* ATCC 30031, *Aspergillus brasiliensis* ATCC 16404.

Suspensions were adjusted to 1x107 CFU/ml (equivalent to 0.5 Mc Farland). These strains were maintained on slants of Nutrient Agar for bacteria and Sabouraud Agar for the fungus at 4°C.The tested microbial strains were provided by the Pasteur Institute (Algiers, Algeria).

Antimicrobial activity

The antimicrobial activity of essential oils from *P. harmala* leaves was evaluated using the paper disk diffusion method (Davis et al., 1994). Petri dishes (90 mm in diameter) were prepared with 20 ml of sterile Mueller-Hinton agar and dimethyl sulfoxide (DMSO) solution for bacteria and Sabouraud agar for fungi. Agar plates were stored at 4°C before use (Fauchère et al., 2002).

The antimicrobial activity test

Saturated with essential oil, the impregnated discs (with 3 µl of essential oil at a final concentration of 1-20µg/ml) were then placed on the surface of the agar (Dean et al., 1987). The bacteria were spread over the discs. , the spreading of the tested product over the entire disc determined the degree of concentration, the micro-organisms grew over the entire surface of the agar except where they encountered a sufficient concentration of the product, which would inhibit their growth after incubation 24h at 37°C (bacteria) and 48h at 25°C (fungi) around of the discs, we observed a clear circular zone formation. This is the zone of inhibition. In addition, this zone of diameter is large; the sensitivity of antibiotics induces more resistance to the bacterium. The diameter of the growth inhibition zone was measured with a calliper and expressed in mm (including the diameter of the 6 mm disc). (Billerbeck et al., 2002; NIST , 2002)

The minimum inhibition concentrations (MICs)

Were performed according to the Muller-Hinton broth microdilution method in 96 multiwall microtiter plates (Marino et al.,2001).The essential oils were dissolved in aqueous DMSO, and the initial concentration was 25µg/ml. (Smânia et al., 1995). The initial concentration was serial dilution, which is a two-fold serial dilution. Each well contained 10^7 CFU/ml of bacteria. After incubation for 24h at 37°C (bacteria) and 48h at 25°C (fungi), the CIM was determined as the lowest concentration at which no visible microorganism growth had occurred. All tests were performed in triplicate (Gilles et al., 2010).

Results and Discussion

The physicochemical analysis of the leaf extracts of *P. harmala* demonstrated the following results: an oily appearance, a yellowish colour, and a strong, unpleasant odour. The yield of essential oil obtained by hydro-distillation of dry plants was 0.217 ± 0.03% for the *P. harmala* Bougtob region and 0.211 ± 0.05% for the Mechria region sample. In a study by Ida et al. in the Bousaada region (South of Algeria), hydrodistillation yielded 0.005% (Ida et al., 2016).

The variations found in the yield of essential oil view of our samples compared to some previous studies may be due to some environmental factors (such as soil composi-

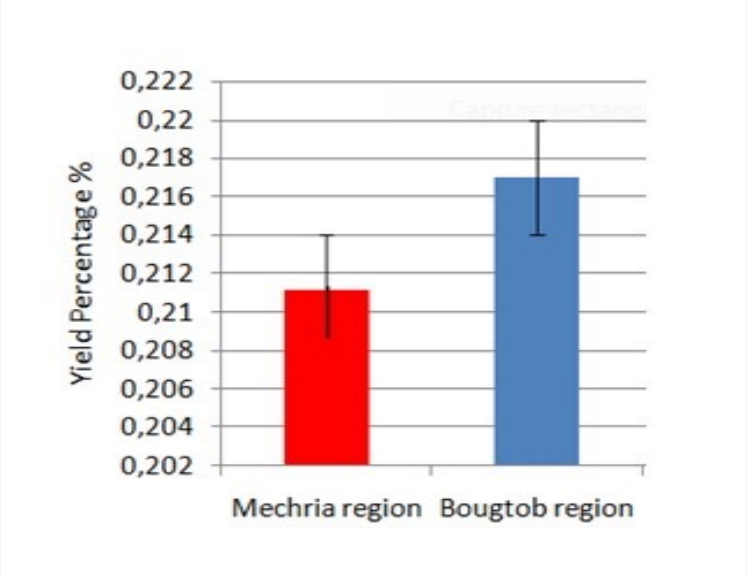


Figure 3. The yield of essential oil of *P.harmala* was obtained by hydro-distillation for different regions.

Slika 3. Donos eteričnega olja *P.harmala*, pridobljenega s hidrodestilacijo za različne regije.

tion, geographical location and climate conditions), the part of the plant used, the age of the plant and the period of the growing season or even to genetic factors (Hussain et al., 2009; Anwar et al., 2009).

Phytochemical screening

Phytochemical screening in the leaves of *P.harmala* showed the presence of flavonoids, alkaloids, saponins, tannins, glycosides, terpenoids and steroids and the absence of anthraquinones for two regions.

Phytochemical Compounds of Essential Oil

The chromatographic analysis of the essential oil of P.harmala from the Bougtob region revealed the presence of 55 volatile, constituting 66.59% of the total essential oil recovered from dry material (Table 4, 5). The chemical analysis showed that the characterized essential oil possessed many potentially bioactive substances, which were identified, and the following compounds are representing the major constituents: (1) Carvone 21.15%, (2) Phytol 17.41%, (3) Carbamic acid 3.52%, (4) Lauric acid 2.10%, (5) Thymol

Table 1. Physicochemical composition of *Peganum harmala*.

Tabela 1. Fizikalno-kemijska sestava *Peganum harmala*.

Specification	Peganum harmala (M)	Peganum harmala (B)
Density	0.905	0.907
Rotating power	+2°	+ 2°
Refractive index	+1.4693	+ 1.4695
Freezing point	-18	-18
Solubility in éthanol 90%	1 :3	1 :3
pH	6.02	6.06
Acid index	0.517	0.521
Ester index	22.7	23.2

(M): Mechria region, (B): Bougtob region.

Table 2. Phytochemical screening of *Peganum harmala* (leaves) hydromethanolic extracts (MM: hydromethanolic extract of Mechria region, MB: hydromethanolic extract of Bougtob region).

Tabela 2. Fitokemijski pregled hidrometanolnih izvlečkov *Peganum harmala* (listi) (MM: hidrometanolni izvleček regije Mechria, MB: hidrometanolni izvleček regije Bougtob).

Extract constituent	Test	Observation	Extract	
			MM	MB
Steroids	Liebermann–Burchard reaction	Purple to blue colour	+	+
Saponins		Foam on the surface	++	++
Tannins		Green color	+++	++
Quinones	Borntragers test		-	-
Phenols		Green-black	+	+
Coumarin glycosides		Green to yellow	++	++
Free anthraquinones		Red or pink colour	-	-
Combined anthraquinones		Pink, red or purple	-	-
Flavonoids		Pink-red or yellow	+	+
Alkaloids	Mayer	Colored precipitate	+	+
Cardiac glucosides		Brown ring	++	++
Proteins	Biuret test	Purple or pink colour	-	-

(-) absence, (+) presence, (+++) presence in high quantity.

1.77%, (6) Tetrapentacontane 1.32%, (7) Dihydroactinidiolide 1.21%, (8) 2-Aminobenzaldehyde 1.03%. In contrast, the chromatographic analysis of the essential oil of P.harmala from the Mechria region demonstrated the presence of 47 volatile, constituting 43.6% of the total essential oil recovered from dry material. Chemical analysis of essential oil from Mechria region showed that its major products are: (1) Phytol 10.24%, (2) Carvone 5.92%, (3) Tetrapentacontane 2.80%, (4) Phytone 2.18%, (5) Butylated Hydroxytoluene 1.48%, (6) Lauric acid 1.39%, (7) Hexatriacontane 1.27%, (8) 3,5-di-tert-Butyl-4-hydroxybenzaldehyde 1.14% .

In a study by Ida et al.(2016), the chromatographic analysis of the essential oil of P.harmala from the Bousaada region demonstrated the presence of 45 volatile constituting 89% of total essential oil showed that its major products are Eugenol 17.5%, Thymol 7%, Carvone 2.7%.

The variations found in the chemical composition from the qualitative and quantitative point of view of our samples compared to the study by Ida et al.(2016) may be due to some environmental factors (such as soil composition, geographical location and climate conditions), the part of the plant used, the age of the plant and the period of the growing season or even to genetic factors (Hussain et al., 2009; Anwar at al., 2009). The methods that use water can induce hydrolysis of esters and also rearrangements, isomerizations, racemizations, and oxidations (Bruneton et al., 1993).

Khadhri et al.(2011) also say that the variation in the percentage of the chemical composition of essential oils can be explained by the differences in the geographic origins, climate, nature of the soil, solar radiation, age of the plant and part of plants.

All these factors involve the activation or inactivation of certain enzymatic groups, leading to the predominance of a particular biosynthetic pathway (Khadhir et al., 2011).

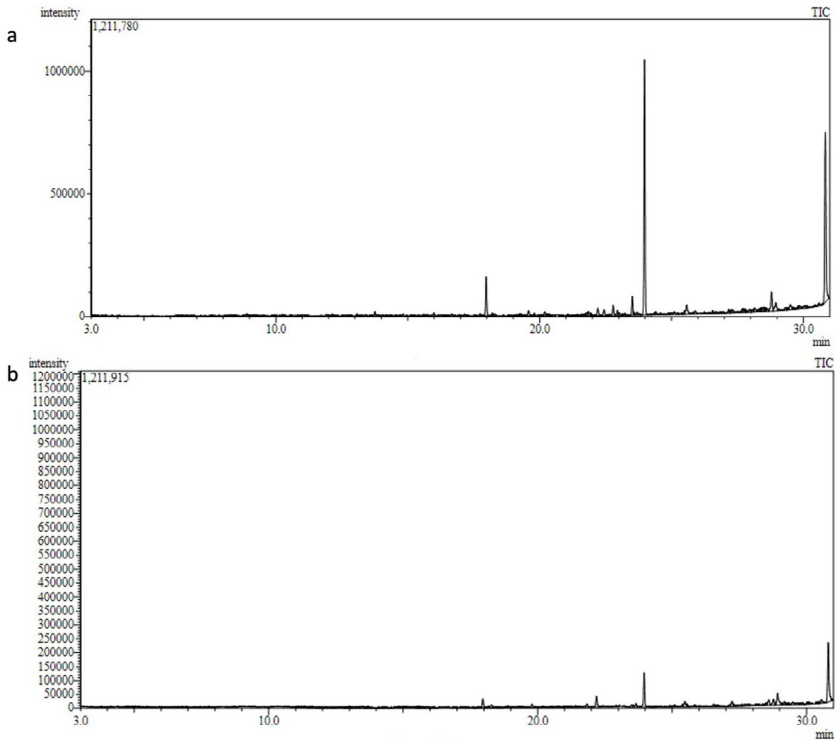


Figure 3. Gas chromatographic profiles the major constituents of *Peganum harmala* essential oils for two regions; a) Gas chromatographic profiles the major constituents of *Peganum harmala* essential oils for Bougtob region; b) Gas chromatographic profiles the major constituents of *Peganum harmala* essential oils for Mechria region.

Slika 3. Profili plinske kromatografije glavnih sestavin eteričnih olj *Peganum harmala* za 2 regiji; a) Profili plinske kromatografije glavnih sestavin eteričnih olj *Peganum harmala* za regijo Bougtob; b) Profili plinske kromatografije glavnih sestavin eteričnih olj *Peganum harmala* za regijo Mechria.

Table 3. Phytochemical components identified in *Peganum harmala* essential oil (Bougtob region) by GC-MS.

Tabela 3. Fitokemične sestavine, identificirane v eteričnem olju *Peganum harmala* (regija Bougtob) z GC-MS.

Compound Name	**Molecular Formula**	**RT (min)**	**Area (%)**
Eicosane	$C_{20}H_{42}$	13.393	0.30
D - carvone	$C_{10}H_{14}O$	13.758	0.53
Anisole	$C_{10}H_{12}O$	15.986	0.36
5,9-undecided-2-one, 6,10-dimethyl-, (e)-	$C_{13}H_{22}O$	16.655	0.31
Heptadecane, 8-methyl-	$C_{18}H_{38}$	17.742	0.22
Carbamic acid	$C_{17}H_{27}NO_2$	17.973	3.52
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	18.295	0.24
Caryophyllene oxide	$C_{15}H_{24}O$	18.816	0.13
Ketone	$C_8H_{12}O$	19.287	0.18
Ethanone, 1-(1,3a,4,5,6,7-hexahydro-4-hydroxy-3,8-dimethyl-5-azulene)-	$C_{14}H_{20}O_2$	19.584	0.56
Hexatriacontane	$C_{36}H_{74}$	19.807	0.25
6,8-nonadien-2-one, 6-methyl-5-(1-methyl ethylidene)-	$C_{13}H_{20}O$	20.196	0.33
2-Benzyl-3-oxo propanoic acid, ethyl ester	$C_{13}H_{16}O_3$	20.305	0.20
P-Cymen-7-ol	$C_{10}H_{14}O$	21.670	0.23
Tetrapentacontane	$C_{54}H_{11}0$	21.768	0.31
2,6-di(t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadiene-1-one	$C_{15}H_{24}O_2$	21.847	0.43
Phytone	$C_{18}H_{36}O$	22.205	0.82
Δ-Cadinene	$C_{15}H_{24}$	22.438	0.67
2-aminobenzaldehyde	C_7H_7NO	22.786	1.03
Eugenol	$C_{10}H_{12}O_2$	22.944	0.46
Thymol	$C_{10}H_{14}O$	23.514	1.77
Hexacontane	$C_{60}H_{122}$	23.695	0.32
D-(+)-carvone	$C_{10}H_{14}O$	23.978	21.15
Ar-tumerone	$C_{15}H_{20}O$	24.401	0.27
Octaborane	B_8H_{12}	24.955	0.14
2-Nonen-1-ol, 2-methyl-	$C_{10}H_{20}O$	25.000	0.18
Decanoic acid	$C_{10}H_{20}O_2$	25.110	0.32
Pentadecane, 8-hexyl-	$C_{21}H_{44}$	25.495	0.31
Dihydroactinidiolide	$C_{11}H_{16}O_2$	25.578	1.21
Quinoline, 6-butyl-	$C_{13}H_{15}N$	25.818	0.19
Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{17}H_{30}OSi$	25.901	0.36
Dihydroartemisinin, 6-dehydro-5-dehydroxy-3-deoxy-	$C_{15}H_{22}O_4$	26.246	0.18
Diethyl phthalate	$C_{12}H_{14}O_4$	26.552	0.26
Solasonine	$C_{45}H_{73}NO_{16}$	26.671	0.19
2-propenoic acid, 3-(dimethylamino)-3-ethoxy-, ethyl ester	$C_7H_{14}O_3$	26.954	0.19
P-vinyl phenol	C_8H_8O	27.174	0.39
Tetracosane	$C_{24}H_{50}$	27.260	0.34
4-quinoline carboxaldehyde	$C_{10}H_7NO$	27.315	0.35
Formamide, N-phenyl-	C_7H_7NO	27.695	0.40
Indole	C_8H_7N	27.782	0.32
5.alpha.-cholestan-6.beta.-amine, n,n-dimethyl-	$C_{29}H_{53}N$	27.962	0.31
Methanone, diphenyl-	$C_{13}H_{12}N$	28.154	0.48
N-Hexadecanoic acid	$C_{16}H_{32}O_2$	28.385	0.24

Ethyl ether	$C_4H_{10}O$	28.501	0.48
Phosphorochloridothioic acid, O,O-dimethyl ester	$C_2H_6ClO_2PS$	28.570	0.19
3,5-di-tert-Butyl-4-hydroxybenzaldehyde	$C_{15}H_{22}O_2$	28.615	0.29
Lauric acid	$C_{12}H_{24}O_2$	28.791	2.10
Tetrapentacontane	$C_{54}H_{110}$	28.950	1.32
3-methylheptadecane	$C_{18}H_{38}$	29.328	0.30
1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester	$C_{22}H_{34}O_4$	29.500	0.75
21.xi-methyl-17-isocholest-16-en-3.Beta.-ol	$C_{27}H_{46}O$	29.848	0.35
Triethylene glycol mono dodecyl ether	$C_{18}H_{38}O_4$	30.058	0.44
Cyclohexanepropanol, .alpha.,2,2,6-tetramethyl-	$C_{13}H_{26}O$	30.144	0.25
Tetracosane	$C_{24}H_{50}$	30.595	0.33
Phytol	$C_{20}H_{40}O$	30.833	17.41

Table 4. Phytochemical components were identified in the *Peganum harmala* essential oil Mechria region by GC-MS.

Tabela 4. Fitokemične sestavine, identificirane v eteričnem olju *Peganum harmala* Mechria regija z GC-MS.

Compound Name	**Molecular Formula**	**RT (min)**	**Area (%)**
Thiirane, octyl-	$C_{10}H_{20}OS$	14.355	0.38
Phosphoric acid	$C_{15}H_{41}O_6PSi_4$	14.962	0.36
Pentacosane	$C_{25}H_{52}$	15.602	0.33
Methyl 2,6-anhydro-.alpha.-d-altroside	$C_7H_{12}O_5$	15.853	0.52
Butanedioic acid, decyl phenylmethyl ester	$C_{21}H_{32}O_4$	17.162	0.38
2-Methyl-decahydro-4-quinolone	$C_{10}H_9NO$	17.268	0.29
Butylated Hydroxytoluene	$C_{15}H_{24}O$	17.960	1.48
β-Ionone	$C_{13}H_{20}O$	18.182	0.38
2-Butenoic acid	$C_{18}H_{27}NO_5$	18.288	0.69
2-Nonadecene	$C_{19}H_{38}$	19.147	0.38
Cyclobutaneacetamide, N-(4-methoxyphenyl)	$C_{10}H_{13}NO_2$	19.288	0.34
Citronellyl valerate	$C_{15}H_{28}O_2$	19.427	0.43
Eicosane	$C_{20}H_{42}$	19.780	0.63
o-Cymene	$C_{10}H_{14}$	20.255	0.24
Hexane, 2,3,4-trimethyl	C_9H_{20}	20.529	0.23
Phenanthrene, 3-methyl-	$C_{15}H_{12}$	20.785	0.57
Propanoic acid, ethyl ester	$C_5H_{10}O_2$	21.060	0.21
Pentanoic acid, 5-hydroxy-2-propyl	$C_{14}H_{32}O_3Si_2$	21.206	0.23
Perhydro-htx-2-one, 2-depentyl-, acetate ester	$C_{16}H_{27}NO_3$	21.411	0.23
13-Methyltritriacontane	$C_{34}H_{70}$	21.735	0.25
2,6-Di(t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	$C_{15}H_{24}O_2$	21.833	0.62
Phytone	$C_{18}H_{36}O$	22.189	2.18
Carvacrol	$C_{10}H_{14}O$	23.499	0.46
Phytol acetate	$C_{22}H_{42}O_2$	23.567	0.37
Tetrapentacontane	$C_{54}H_{110}$	23.657	0.71
Carvone	$C_{10}H_{16}O$	23.960	5.92
Octadecanoic acid	$C_{18}H_{36}O_2$	25.090	0.33
Hexatriacontane	$C_{36}H_{74}$	25.472	1.27
2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)-	$C_{11}H_{16}O_2$	25.562	0.37
Quinoline, 6-butyl-	$C_{13}H_{15}N$	25.828	0.32

α-Pyrrolidone, 5-[2-butyrylethyl]-	$C_{10}H_{17}NO_2$	26.016	0.42
Malonic acid, 6-heptynyl	$C_{15}H_{28}O_4$	26.285	0.39
1,2-Benzenedicarboxylic acid, diethyl ester	$C_{12}H_{14}O_4$	26.541	0.63
Dotriacontane	$C_{32}H_{66}$	27.233	1.13
Spiro[cyclopropane-1,2'(1'h)-phenanthrene]-1',4'(3'h)-dione, 8'-[(formyloxy) methyl]-4'b,5',6',7',8',8'a,9',	$C_{21}H_{28}O_7$	27.281	0.34
1,3,2-Dioxaphosphorinane, 2-(benzyloxy)-2-oxo-	$C_{10}H_{13}O_4P$	27.811	0.57
2-(2-Bromo-ethyl)-3-methyl-oxirane	C_5H_9BrO	28.377	0.23
3-Quinuclidinol	$C_7H_{13}NO$	28.508	0.47
3,5-di-tert-Butyl-4-hydroxybenzaldehyde	$C_{15}H_{22}O_2$	28.600	1.14
Lauric acid	$C_{12}H_{24}O_2$	28.773	1.39
Benzenamine, 2,4-dinitro-N-phenyl-	$C_{12}H_9N_3O_4$	28.840	0.36
Tetrapentacontane	$C_{54}H_{110}$	28.924	2.80
Phosphonic acid, bis(1-methylethyl) ester	$C_6H_{15}O_3P$	29.059	0.40
Pentadecane, 2,6,10-trimethyl-	$C_{18}H_{38}$	29.181	0.55
1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester	$C_{22}H_{34}O_4$	29.477	0.68
Tetrapentacontane	$C_{54}H_{110}$	30.554	0.76
Phytol	$C_{20}H_{40}O$	30.808	10.24

Antibacterial activity

The essential oil of P.harmala leaves of two regions has good inhibitory activity in vitro against the microorganisms tested. However, the microorganisms did not show the same sensitivity to the essential oil.

This essential oil has strong antibacterial activity against Gram-positive bacteria, Staphylococcus aureus (inhibition zone 36 mm MIC: 10.5 µg/ml and 33 mm MIC: 11.5 µg/ml) and Bacillus cereus, (inhibition zone 21.7 mm MIC: 15 µg/ml and 18 mm MIC: 17.5 µg/ml) and very strong antifungal, Candida albicans (inhibition zone 25 mm MIC: 09.0 µg/ml and 22.5 mm MIC: 11.5 µg/ml) and Aspargillus brasiliensis (inhibition zone 18 mm MIC: 7.5 µg/ml and 17.5 mm MIC: 10.5 µg/ml). The essential oils have moderate antibacterial activity against Gram-negative bacteria, Pseudomonas aeruginosa (inhibition zone 10.5 mm MIC: 19.5 µg/ml and 8.5 mm MIC: 21 µg/ml), Klebsiella pneumonia (inhibition zone 13.5 mm MIC: 17.5 µg/ml and 12 mm MIC: 18.5 µg/ml), Escherichia coli (inhibition zone 17 mm MIC: 14.5µg/ml and 15.5 mm MIC: 12 µg/ml) due to the presence of lipopolysaccharide barrier (LPS), which has a role in protecting bacteria against antibacterial agents.

Gram-negative bacteria are more resistant to essential oils than Gram-positive bacteria due to the structure of their outer membrane. Thus, the outer membrane of Gram-negative is richer in lipo-polysaccharides and proteins (Toure et al., 2015).

Conclusions

In this study, we determined the chemical composition and the antimicrobial activity of essential oil from the leaves of *Peganum harmala* grown in the region of Bougtob and Mechria. Fifty-five compounds havebeen identified in the essential oil of the sample of the first region, Carvone 21.15%, Phytol 17.41%, Carbamic acid 3.52%, Lauric acid 2.10%, and Thymol 1.77%, Tetrapentacontane 1.32% were the major compounds. In the sample of the region of Mechria, 47 compounds were identified when Phytol 10.24%, Carvone 5.92%, Tetrapentacontane 2.80%, Phytone 2.18%, Butylated Hydroxytoluene 1.48%, and Lauric acid 1.39% were the dominant.This difference in composition is due to several factors, such as soil composition, geographical location and climate conditions.

Phytochemical screening in the leaves of *P.harmala* showed the presence of flavonoids, alkaloids, saponins, tannins, glycosides, terpenoids and steroids and the absence of anthraquinones.

The antimicrobial effectiveness of the essential oil of *P.harmala* from southern Algeria has been demonstrated against five bacteria and fungi. This essence showed strong antimicrobial activity against all the tested strains. In general, Gram-positive bacteria and fungi were found to be more sensitive to the essential oils than Gram-negative bacteria. This great power is attributed mainly to their high

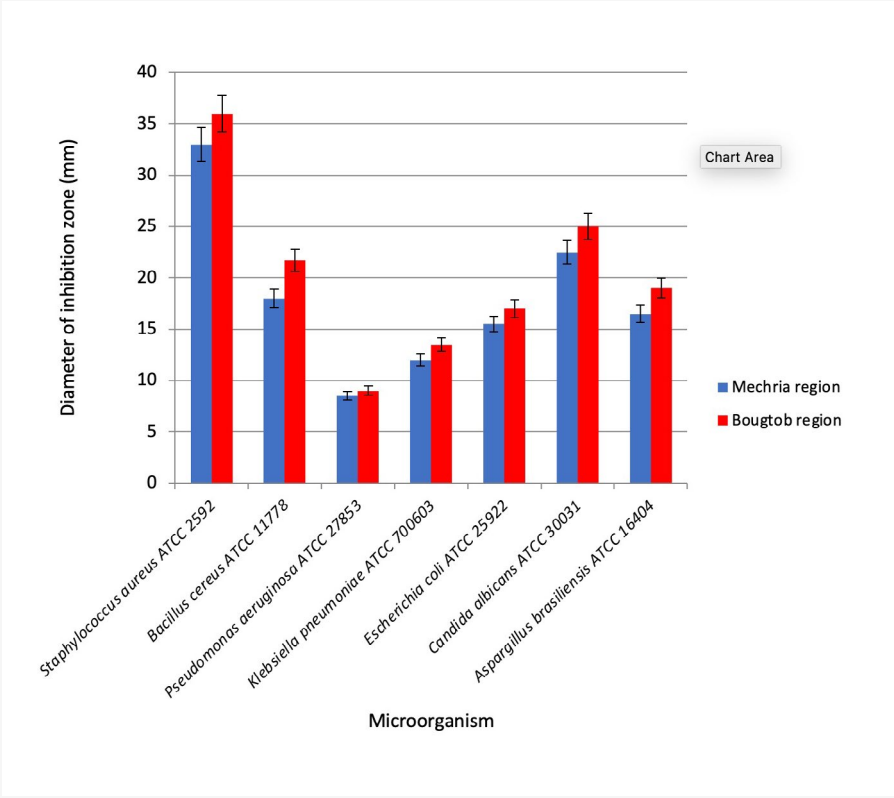


Figure 4. Zones of growth inhibition (mm) are used using a direct contact technique in an agar medium for the antimicrobial activity of *P.harmala* essential oils.

Slika 4. Območja zaviranja rasti (mm) so s tehniko neposrednega kontakta v agarskem mediju protimikrobna aktivnost eteričnih olj *P.harmala.*

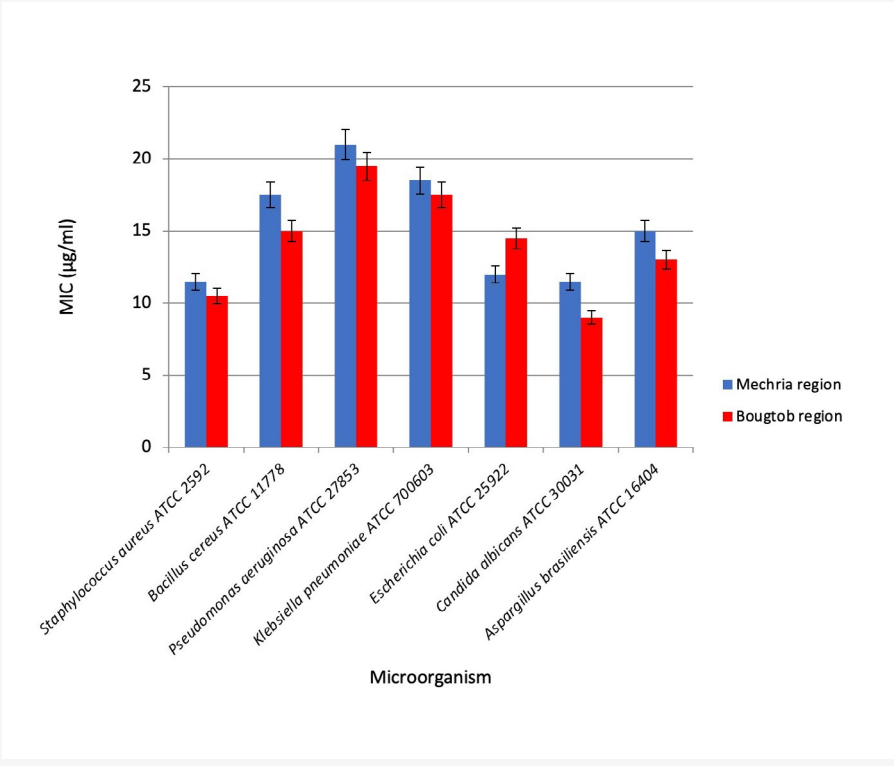


Figure 5. MIC (µg/ml) using microdilution method in 96 multiwall microliter plates showing antimicrobial activity of *P.harmala* essential oils.

Slika 5. MIC (µg/ml) z metodo mikrorazredčevanja v 96 večstenskih mikrolitrskih ploščah, ki prikazuje protimikrobno delovanje eteričniholj *P.harmala.*

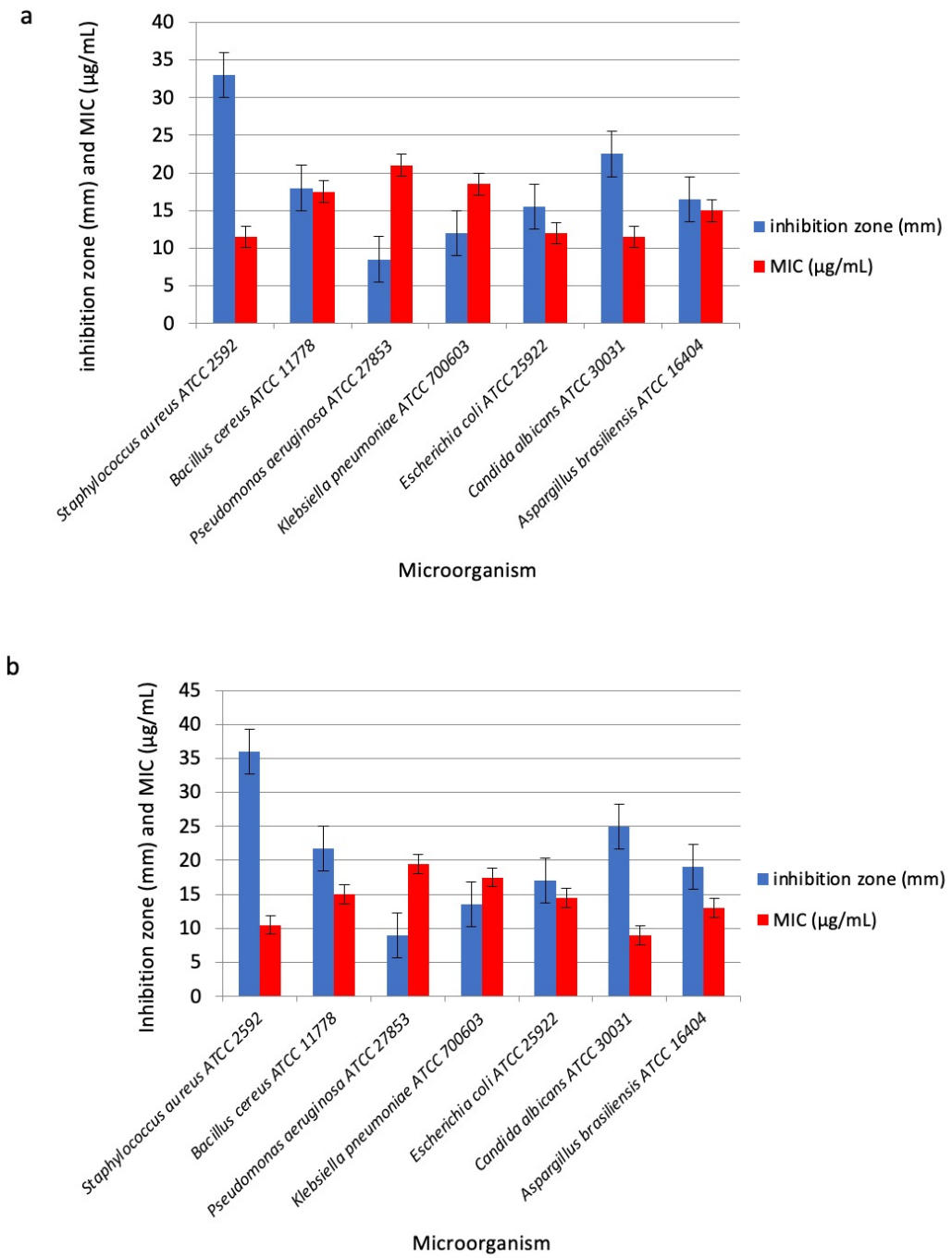


Figure 6. Comparison of Diameter ofinhibition zone and MIC for the individual bacteria/fungi fortwo regions; a. Comparison of Diameter ofinhibition zone and MIC for the individual bacteria/fungi for Mechria region; b. Comparison of the Diameter of the inhibition zone and MIC for the individual bacteria/fungi for the Bougtob region

Slika 6. Primerjava premera inhibicijskega območja in MIC za posamezne bakterije/glive za 2 regiji; a. Primerjava premera inhibicijskega območja in MIC za posamezne bakterije/glive za regijo Mechria; b. Primerjava premera inhibicijskega območja in MIC za posamezne bakterije/glive za regijo Bougtob

contents in oxygenated terpenes (Carvone, Phytol and Lauric acid). However, the antimicrobial activity registered confirms some of the traditional uses of the plant in the treatment of some diseases.

As a final remark, it is also vital to mention that the essential oil of *Peganum harmala* is a domain where much remains to be studied to exploit its properties.

Author Contributions

Conceptualization, G.A. and C.O.; methodology, G.A. and C.O.; software, G.A.; validation, G.A., C.O., K.F. and F.Z.; formal analysis, F.Z.; investigation, C.O. and K.F.; data curation, G.A., C.O., K.F., F.Z., and D.Z.; writing original draft preparation, G.A., C.O., K.F., F.Z., and D.Z.; writing review and editing, G.A., C.O., K.F., F.Z., and D.Z.; visualization, C.O; supervision, C.O; project administration, C.O. All authors have read and agreed to the published version of the manuscript.

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Data Availability

Data are available upon request to the corresponding author.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could haveappeared to influence the work reported in this paper.

References

Abodunrin, T., Uhuegbu, C., and Olugbuyiro, J., 2015. Phytochemical analysis of leaf-extracts from eight tropical trees: Prospects for environmentally-friendly dye compounds for smart windows. International Journal of Scientific and Engineering Research, 6(3), 682-698.

Adou, L.M.D., Kone, M.W., Ipou, J.I., N'guessan, E.K., 2016. Ethnobotanique et analyse phytochimique qualitative de *Pteridium aquilinum* (L.) Kühn (Dennstaedtiaceae), une Ptéridophyte utilisée comme plante médicinale en Côte d'Ivoire. International Journal of Biological and Chemical Sciences, 10(4), 1783-1792. https:// doi.org/10.4314/ijbcs.v10i4.27.

AFNOR. Huiles essentielles. Ed. PARA Graphic. Tome1 – Echantillonnage et méthode d'analyse 471 P. Tome 2 – Volume 1 Monographie relative aux huiles essentielles Tome 2 – Volume 2 Monographie relative aux huiles essentielles 2000; 663 pp.

Amezouar, F., Badri, W., Hsaine, M., Bourhim, N., Fougrach, H., 2013. Antioxidant and anti-inflammatory activities of Moroccan *Erica arborea* L. Pathologie-Biologie, 61(6), 254-258.https://doi.org/10.1016/j. patbio.2013.03.005

Anwar, F., Ali, M., Hussain, A.I., Shahid, M. 2009. Antioxidant and antimicrobial activities of essential oils and extracts of fennel (*Foeniculum vulgare* Mill.) seeds from Pakistan. Flaveur and Fragrance Journal, 24, 170-176.

Bahmani, M., Rafieian-kopaei, M., Parsaei, P., Mohsenzadegan, A. 2012. The anti-leech effect of *Peganum harmala* L. extract and some anti-parasite drugs on *Limnatis nilotica*. African Journal of Microbiology Research, 6(10): 2586-2590.

Billerbeck, V.C., Roques, C., Vaniére, P., Marquier, P.,2002. Activité antibactérienne et antifongique des produits à bases d'huiles essentielles.Hygienes, 3, 248-251.

Boutekedjiret, C., Bentahar, F., Belabbes, R.,Bessiere, J.M., 2003. Extraction of rosemary essential oil by steam distillation and hydrodistillation. Flavour andFragrance Journal, 18, 481-484.

Bouzouita, N., Kachouri, F., Ben Halima, M., Chaabouni, M.M., 2008. Composition chimique et activités antioxydante, antimicrobienne et insecticide de l'huile essentielle de Juniperusphoenicea. Journal de la Société Chimique de Tunisie, 10, 119-125.

Bruneton, J., 1993. Pharmacognosy, Phytochemistry medicinal plants. Technical and documentary, 3rd edition., 634 pp.

Centers for Disease Control and Prevention, 2013. Office of Infectious Disease Antibiotic resistance threats in the United States, Apr 2013.

Chouitah, O., 2017. Essential oil from the leaves of *Ajuga iva*: Chemical Composition and Antimicrobial Activity, Journal of Essential Oil Bearing Plants, 20(3), 873-877.

Davis, J., 1994. Inactivation of antibiotics and the dissemination of resistance genes. Science, 246, 375-382.

Dean, S.G., Ritchie, G., 1987. Antibacterial properties of plant essential oils.International Journal Food Microbiology, 5, 165-180.

Fauchère, J.L., Avril, J.L., 2002. General and medical bacteriology. Ellipses Editions Paris, 365 pp.

Gilles, M., Zhao, J., An, M., Agboola, S., 2010.Chemical composition and antimicrobial properties of essential oils of three Australian Eucalyptus species.Food Chemistry. 119: 731–737.

Haddouchi, F., Benmansour, A., 2008. Huiles essentielles, utilisation et activités biologiques Application à deux plantes aromatiques. Les technologies de laboratoires, 3(8), 20-27.

Haddouchi, F., Chaouche, T., Halla, N., 2016. Screening phytochimique, activités antioxydantes et pouvoir hémolytique de quatre plantes sahariennes d'Algérie. Phytothérapie, 1-9. https://doi.org/10.1007/s10298-016-1086-8

Hossain, M.A., Al-raqmi, K.A.S., AL-mijizy, Z.H., Weli,A.M., AL-Riyami,Q., 2013. Study of total phenol, flavonoids contents and phytochemical screening of various leaves crude extracts of locally grown *Thymus vulgaris*. Asian Pacific journal of tropical biomedicine, 3(9), 705- 710. https://doi.org/10.1016/S2221-1691(13)60142-2

Hussain, A.I., 2009. Characterization and biological activities of essential oils of some species of Lamiaceae. Doctorate thesis in chemistry. University of agriculture, Faisalabad,Pakistan, 257 pp.

Ida, A., Luigi, A., Lucia, C., Vincenzo, D., Florida, F., Filomena, N., Lucèia, F.S., Maroua, K., 2016. Chemical composition, antibacterial and phytotoxic avtivites of *Peganum harmala* seed essential oils from five different localities in Northern African Journal of Molecules,21,1235.

Ismail, A.M., Mohamed, E.A., Marghany, M.R., Abdelmotaal, F.F., Abdel-farid, I.B., EL-sayed, M.A., 2016. Preliminary phytochemical screening, plant growth inhibition and antimicrobial activity studies of *Faidherbia albida* legume extracts. Journal of the Saudi Society of Agricultural Sciences, 15(2), 112-117.https://doi.org/10.1016/j.jssas.2014.06.002

Joshi, A., Bhobe, M., and Sattarkar, A., 2013. Phytochemical investigation of the roots of *Grewia microcosLinn*. Journal of Chemical and Pharmaceutical Research, 5(7), 80-87.

Khadhir, A., El Mokni,R., Mguis,K., Ouefelli, I., Araújo, M.E.M., 2011. Variability of two essential oils of *Ammivisnaga* (L.) Lam. a traditional Tunisian medicinal plant. Journal of Medicinal Plants Research, 5(20), 5079-5082.

Kumar, B.R., 2015. Preliminary test of phytochemical screening of crude ethanolic and aqueous extract of *Moringa pterygosperma* Gaertn. Journal of Pharmacognosy and Phytochemistry, 4(1), 7-9.

Maria, R., Shirley, M., Xavier, C., Jaime, S., David, V., Rosa, S., Jodiea, D., 2018.Preliminary phytochemical screening, total phenolic content and antibacterial activity of thirteen native species from Guayas province Ecuador. Journal of King Saud University-Science, 30(4), 500-505. https://doi.org/10.1016/j.jksus.2017.03.009.

Marino, M., Bersani, C., Comi, G., 2001. Impedance measurements to study the antimicrobial activity of essential oils from Lamiaceae and Compositae. International Journal of Food Microbiology, 67(3), 187-95.

Marwat, S.K., ur Rehman, F., 2011.Chapter 70 - Medicinal and Pharmacological Potential of Harmala (*Peganum harmala* L.) Seeds. In: Reedy, V.R., Watson, R., Patel, V.B. (Eds.) Nuts and Seeds in Health and Disease Prevention. London: Academic Press, pp. 585-599.

NIST National Institute of Standard Library, 2002. The Perkin Eime Corporation. NCCLS. Performance Standards for Antimicrobial Susceptibility Testing. National Committee for Clinical Laboratory Standards, Wayne, Pennsylvania, USA, Twelfth International Supplement; M100-S12.

Pandey, M.M., Rastogi, S., Rawat, A.K.S., 2013. Indian traditional ayurvedic system of medicine and nutritional supplementation. Evidence-Based Complementary and Alternative Medicine, 2013, 1-12.

Quezel, F., Santa, S.,1963. Nouvelle Flore de l'Algérie et des régions désertiques méridionales. Vol. 1-2. Ed. CNRS, Paris France.

Rashid, C.A., Qureshi, M.Z., Raza, S.A., William, J., Arshad, M., 2010. Quantitative determination of antioxidant potential of *Artemisia persica*, Analele University Bucureşti - Chimie, 19(1), 23-30.

Read, A.F., Woods, R.J., 2014. Antibiotic resistance management. Evolution, Medicin, and Public Health, 2014(1):147. doi: 10.1093/emph/eou024.

Roosta, R.A., Moghaddasi, R., Hosseini, S.S., 2017. Export target markets of medicinal and aromatic plants. Journal of applied research on medicinal and aromatic plants, 7, 84-88.

Rudramurthy, G.R., Swamy, M.K., Sinniah, U.R., Ghasemzadeh, A., 2016. Nanoparticles: alternatives against drug-resistant pathogenic microbes. Molecules, 21(7), 7-8.

Samarth, R.M., Samarth, M., Matsumoto, Y., 2017. Medicinally important aromatic plants with radioprotective activity. Future Science OA, 3(4): 1-26.

Smânia, A., Monache, F.D., Smânia, E.F., Gil, M.L., Benchetrit, L.C., Cruz, F.S., 1995. Antibacterial activity of substance produced bythe fungus *Pycnoporus sanguineus* (Fr.) Murr. Journal of Ethnopharmacology, 45(3), 177-81.

The antibiotic alarm. Nature 2013, 495(7440):141. doi: 10.1038/495141a.

Toure, D., 2015. Etude chimique et biologique des huiles essentielles de quatre plantes aromatiques medicinales de Cote d'Ivoire. Doctorate thesis in biochemistry. University of Felix Houphouët-Boigny.

Zargari, A., 1992. Medicinal Plants. Teharan: Tehran University Publication, 448 pp.

ABS

Original Research

Soil mesofauna diversity in agricultural systems of Slovenia using the QBS index and its modifications

Vid Naglič [1*], Nataša Šibanc [2], Tine Grebenc [2], Irena Bertoncelj [1]

Abstract

Soil mesofauna plays a key role in maintaining soil health by supporting the decomposition of organic matter, nutrient cycling and the maintenance of soil structure. In this study of Slovenian agricultural ecosystems, we used four modifications of the QBS index, a soil biological quality index based on soil mesofauna. We compared diversity in arable fields under different tillage intensities, a strawberry field and an orchard, managed with either organic or integrated pest management methods (IPM). The results show significant differences in the mesofaunal communities in the soil. Minimum tillage promoted higher biodiversity, especially of Collembola, compared to conventional tillage. In fruit production systems, the ratio of Collembola to Acarina differed from that of arable fields, skewing in favour of Collembola, possibly related to the use of copper-containing pesticides in organic orchards and systemic herbicides in IPM systems. The QBS index values for soil health varied considerably between systems. Only QBS modifications considering the abundances of organisms (QBS-ab and QBS-a) were able to distinguish between different system-management groups. This study provides insights into the limitations of the originally proposed QBS-ar index to discern the effects of farming intensity on the soil mesofaunal community. Results suggest that minimum tillage and organic management practices can promote healthier soil ecosystems, emphasizing the importance of sustainable soil management for the promotion of soil biodiversity. Future research should aim to incorporate a broader range of agricultural practices and assign fauna to a higher taxonomic rank to further explain the effects on soil mesofauna diversity.

Keywords

Soil health, Soil microarthropods, Biodiversity, Agroecosystems, Tillage intensity, Organic farming

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Raziskava raznovrstnosti talne mezofavne v kmetijskih ekosistemih Slovenije z uporabo QBS indeksa in njegovih izpeljank

Izvleček

Talna mezofavna z opravljanjem ekosistemskih storitev razgradnje organske snovi, kroženja hranil in vzdrževanju strukture tal igra ključno vlogo pri ohranjanju zdravja tal. V tej raziskavi slovenskih kmetijskih ekosistemov smo uporabili štiri različice QBS indeksa, ki so bile razvite za namen preučevanja mezofavne v tleh. Raznolikost te skupine živali smo preučevali na njivah z različnimi intenzivnostmi obdelave tal, v nasadu jagod in sadovnjaku, kjer se uporabljajo ekološke ali integrirane metode varstva rastlin (IPM). Rezultati so pokazali statistično značilne razlike v talni mezofavni preučevanih kmetijskih ekosistemov. Pri minimalni obdelavi tal v primerjavi s konvencionalnim oranjem je bila biodiverziteta višja, zlasti pri skupini Collembola. V sistemih pridelave sadja se je razmerje med Collembola in Acarina v prid Collembola razlikovalo od tistega na njivah, kar je verjetno povezano z večjo občutljivostjo Acarina na bakrove pesticide v ekoloških sadovnjakih in sistemske herbicide v IPM sistemih. Vrednosti QBS indeksa so se med sistemi razlikovale. Le QBS različici, ki upoštevata številčnost organizmov (QBS-ab in QBS-a), sta zaznali razlike med različnimi skupinami sistemov in načinov upravljanja. Ta študija kaže na omejitve prvotno predlaganega QBS-ar indeksa za zaznavanje vplivov intenzivnosti kmetovanja na mezofavno v tleh. Rezultati nakazujejo, da lahko minimalna obdelava tal in ekološko upravljanje spodbujata bolj zdrave talne ekosisteme, kar poudarja pomen trajnostnega upravljanja za spodbujanje biodiverzitete v tleh. Da bi lahko natančneje pojasnili vplive kmetijskih praks na raznolikost talne mezofavne, bi morali v prihodnje raziskave vključiti širši spekter kmetijskih ekosistemov ter določiti talne živali do višjih taksonomskih kategorij.

Ključne besede

Zdravje tal, Talni členonožci, Biodiverziteta, Agroekosistemi, Intenzivnost obdelave tal, Ekološko kmetijstvo

Introduction

The topic of soil health and its indicators has gained considerable research interest in the past 20 years, and as soil mesofauna are responsible for several ecosystem services, they have been proposed as a potential soil quality and soil health indicator (Menta & Remelli, 2020). Soil mesofauna plays a key role, being responsible for numerous essential functions such as organic matter decomposition, nutrient cycling, and soil structure maintenance. As such, they are increasingly recognized as valuable indicators of soil quality and health (Menta & Remelli, 2020). The dominance and diversity of specific taxa, notably *Acarina* (mites) and *Collembola* (springtails), in agricultural soils have been extensively studied (Behan-Pelletier, 2003). These taxa are not only most abundant but also sensitive to environmental changes, with the potential to serve as reliable bioindicators. Previous research (Behan-Pelletier, 2003) has documented different proportions of *Acarina* and *Collembola* in various agricultural settings, highlighting their response to different farming practices. For instance, higher abundance of *Acarina* in organically managed systems compared to integrated pest management (IPM) (Gagnarli et al., 2015) and changes in *Collembola* abundance as a response to different tillage practices (Vignozzi et al., 2019). Among various indices proposed for assessing soil health, there is the QBS (Qualità Biologica del Suolo) index, which is based on the presence of soil arthropod communities and their level of adaptation to living in soil (Parisi et al., 2005). The QBS index has gained considerable attention in some parts of the world, particularly in Italy, where it originated, and has been applied at the regional level (Albertazzi et al., 2021) to highlight the importance of soil biodiversity for ecosystem services (Menta, Conti, Pinto, et al., 2018). What makes this index advantageous is that it does not require species-level identification, making it a practical tool for large-scale ecological assessments and non-taxonomy specialists.

The use of indices for describing ecosystems stems from the challenge of grasping the full complexity of these systems, and such indices may offer a simplified

view aimed at addressing specific research questions. As a result, these indices may not always be effective in detecting significant differences in soil quality between various management practices, especially in ecosystems that are heterogeneous or highly disturbed (Tabaglio et al., 2009). Recent advancements in molecular metabarcoding offer more precise tools for assessing soil biodiversity (Orgiazzi et al., 2015; Guerra et al., 2020). These methods enable more detailed identification, moving from the class-level identification necessary for the QBS index to genus or even species-level identification. Studies confirmed a strong positive correlation between molecular and classical identification tools applied to insects (Jin et al., 2013), and some studies have already been done specifically for soil fauna (Basset et al., 2022). Although genetic studies have provided valuable insights into soil mesofauna, they often miss crucial information about the abundance of these organisms. Additionally, relying solely on DNA analysis may be less effective at detecting recent changes in soil communities, as the persistence of relict DNA from soil organisms can distort our understanding of the current structure of the soil fauna community (Foucher et al., 2020).

The QBS index and its variations are employed to assess the impact of agricultural practices on soil biodiversity and health, a need that has become increasingly important due to the new Soil monitoring law proposed by the EU (General Secretariat of the Council, 2024). In arable fields, the intensity of tillage is a critical factor influencing soil mesofauna. Conventional tillage, which disrupts soil structure, often negatively impacts soil biota, whereas no-till practices tend to enhance soil biodiversity and ecosystem services (Betancur-Corredor et al., 2022). In fruit-growing systems like orchards and strawberry fields, organic farming is generally associated with higher biodiversity due to reduced chemical inputs and a focus on ecological balance. However, the specific contributions of various agricultural practices and soil management approaches influence soil fauna diversity, and consequently, soil health and biodiversity remain unknown.

In this study, we aim to explore the diversity and abundance of soil mesofauna across three different agricultural ecosystems with two contrasting soil management systems by applying four different variants of the QBS index. The QBS-based approach was selected as an affordable and simple approach. The experiment was set in Slovenia, where highly preserved (extensive) agricultural lands and intensive soil management intermix at small spatial scales. Arable fields, strawberry fields, and orchards were selected as test cultures affecting the soil fauna community, with a focus on varying tillage intensities and the distinction between organic and IPM production methods.

Materials and Methods

Study sites

The experiment was conducted at two locations of the Agricultural Institute of Slovenia's field research stations: an arable field at the Infrastructure Centre Jablje (46.141204, 14.571509) and a strawberry field and orchard both located at the Infrastructure Centre Brdo (46.166927, 14.680106). The sampling sites within each location were on the same geological base, namely clay gravel and mixed origin gravel soil for Jablje, and clay gravel, sandy loam and clay for Brdo, respectively; same climate conditions Cfbw' (moderate warm humid climate with warm summers and peak precipitation in one of the autumn months) (Ogrin et al., 2023) according to the Köppen climate classification; with comparable micro-location on flat surface; similar average annual precipitation of 1300-1400 mm and average annual temperatures 10-12°C (ARSO, 2023).

The arable field in Jablje was cultivated for five years prior to sampling (since 2018) using three tillage methods: no-tillage without any soil disturbance (0.43 ha), minimal tillage using a disc cultivator or a ripper to a depth of 8-10 cm (0.96 ha), and conventional tillage with ploughing to a depth of 20-25 cm (0.96 ha). The same three-year crop rotation of winter cereal with a cover crop, maize, and soybeans has been practised for all three tillage methods. Fields were fertilized with mineral fertilizers and adapted to crop requirements with average values of 80-100 kg P_2O_5, 100-130 kg K_2O and 160-180 kg N per hectare. Herbicides were applied on average once per year, and fungicides 2-3 times per year in winter wheat crops. All fields were under a large share of crops in the rotation, and no significant pest damage like maize, crimson clover, or soybeans was observed. For the past 50 years, insecticides were used only occasionally, with the frequency once every 4-5 years. The last insecticide application (7.5 g/ha of Lambda-cyhalothrin) was 2 years ago. Lambda-cyhalothrin half-life is around 30-60 days, and considering soil and environmental conditions at the study site (warm and microbiological active soils), insecticide was degraded in a couple of

months at the latest (Hornsby et al., 1996). The study area was not known to be actively exposed to plastic materials; however, since microplastics and pesticide residues have not been analyzed in this area to date, we cannot rule out their potential presence or influence on mesofauna.

Fruit production at the Infrastructure Center Brdo is based on organic and IPM production methods. The strawberry field was divided into an organic and an IPM section, planted with strawberries (*Fragaria x ananassa* Duch.; cultivar Clery) two years prior to sampling (in August 2021); four ridges were made, covered with black polyethene and equipped with drip irrigation system. The organic section transitioned through various crops before planting strawberries in 2021, where no fertilizers were used and only plant protection products approved for organic production were used (*Pravilnik o ekološki pridelavi in predelavi kmetijskih pridelkov oziroma živil.*, 2018). In the IPM section, where strawberries were grown according to the recommended crop rotation for eight years before sampling (since 2015), systemic herbicides and only a few fungicides were used before planting.

The orchard in Brdo covers 14.9 ha and is also divided into an organic and an IPM section. The Topaz apple variety (*Malus domestica* var. Topaz) has been cultivated according to the rules of organic production since 2009 (*Pravilnik o ekološki pridelavi in predelavi kmetijskih pridelkov oziroma živil.*, 2018) without the use of herbicides. To remove the weed vegetation under the trees, a rotary tiller is used in combination with a weed brush, which mechanically disturbs the soil to a depth of up to 5 cm. For control of diseases, only solutions approved for organic farming were used, which are mostly copper or sulfur-based (Lešnik et al., 2016; *Pravilnik o ekološki pridelavi in predelavi kmetijskih pridelkov oziroma živil.*, 2018). The Gala apple variety (*Malus domestica* var. Gala) has been grown according to IPM management guidelines with biennial herbicide application according to the Technical guidance (*Pravilnik o integrirani pridelavi poljščin, zelenjave, hmelja, sadja in oljk ter grozdja*, 2023) in the herbicide strip under the trees for control of the weeds.

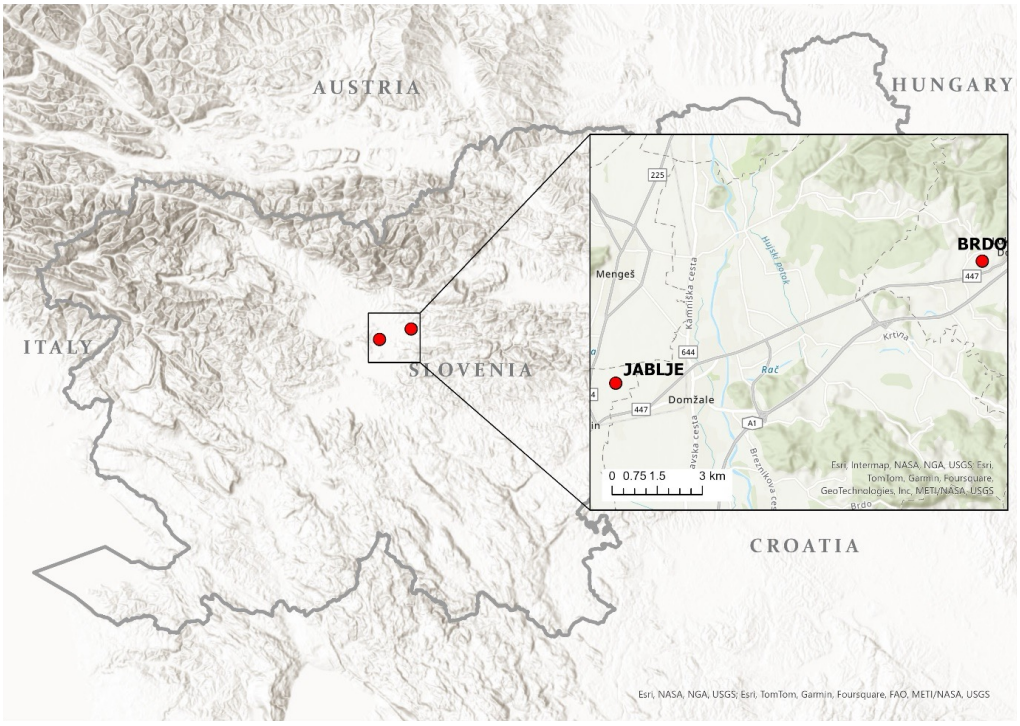

Figure 1. Location of study sites Jablje and Brdo.

Slika 1. Lokacija vzorčnih območij Jablje in Brdo.

Experimental setup and soil mesofauna sampling

Sampling was conducted in three agroecosystems: an arable field, a strawberry field, and an orchard. In the arable field, samples of soil were taken from three tillage methods: no-tillage, minimal tillage, and conventional tillage, with nine samples of each type, giving a total of 27 samples from the arable ecosystem. In the strawberry field, nine samples were taken from each of the organic and IPM sections, giving a total of 18 samples. Two-thirds of the samples were collected from the ridges next to the roots of plants, and one-third of the samples were collected from the interrow spaces between the ridges. In the orchard, we collected nine samples from both the organic and IPM sections, totalling 18 samples, with one-third taken from the root zone of the apple trees and two-thirds from the inter-row areas covered with grass. However, for data analysis, all samples were pooled, resulting in a combined sample size of n=9 for each agricultural system-method group.

Standardized soil samples were taken following the QBS sampling protocol (Parisi et al., 2005) using a soil corer with an 11.3 cm diameter to a depth of 10 cm to collect 1 litre of soil in each soil sample. Prior to sampling, the top layer of vegetation was removed using scissors. The soil was collected in plastic bags and transferred to the Kempson extractor (ecoTech, Bonn, Germany) (Kempson, 1963), where the soil mesofauna was extracted by air drying the samples for 10 days at 30 °C. Extracted animals were preserved in 70% ethanol.

Laboratory analysis of soil mesofauna

Extracted animal individuals were identified and counted using stereomicroscope and classified into taxonomic groups (on the level of class or order) and into eco-morphologic groups according to the protocol established for the calculation of the QBS index (Parisi et al., 2005). Eco-morphological groups are based on taxonomic groups (class or order level), which are assigned an eco-morphological index (EMI) value between 1 and 20 (possible values are 1, 2, 4, 5, 6, 8, 10, 15, and 20) according to their level of adaptation to the soil environment, with higher values indicating higher adaptation to soil life. The level of adaptation is determined by morphological characteristics such as body size, pigmentation, length of appendages and presence of eyes. The total number of eco-morphological groups available according to the QBS methodology is 53. Three categories of eco-morphological groups have been proposed by the QBS index authors: epiedaphic (values 1, 2, 4), hemiedaphic (values 5, 6, 8, 10) and euedaphic (values 15, 20) (Parisi et al., 2005).

QBS-ar was calculated by summing the highest EMI values of taxonomic groups. For example, Collembola were divided into seven eco-morphological groups with EMI values between 1 and 20. For QBS-ar (Parisi et al., 2005), only the highest EMI value recorded in the sample for Collembola was considered. The second modification was QBS-ar BF (D'Avino et al., 2023), which summarized the EMI values of all present eco-morphological groups in the sample. The third modification, named QBS-a (proposed in this article), considered information on abundance, which was ignored by the previous two, by multiplying the EMI value with the abundance of each eco-morphological group in the sample. The result was divided by 100 for readability. The fourth modification, named QBS-ab, was proposed by Mantoni et al. (2021), where the abundance of each eco-morphological group was logarithmically transformed before being multiplied by its EMI value. This was done to reduce the influence of the most abundant groups (Acarina and Collembola).

Data analysis

Cumulative abundance and log-transformed cumulative abundance of taxonomic and eco-morphological groups in all samples were calculated. Species richness rarefaction curves for each agroecosystem were used to determine whether our sampling was thorough with enough collected samples.

Principal component analysis (PCA) was used for soil mesofauna community analysis using QBS eco-morphological groups as species. Sample scores on the first and second PCA axes were compared among the seven system-method groups using ANOVA with Tukey post-hoc tests conducted in R software version 4.4.0 ("stats" library). Average species scores (loadings) of epiedaphic, hemiedaphic and euedaphic groups were calculated.

In our statistical analysis, we categorized our samples into seven system-method groups, each with nine samples: Arable field-conventional (n=9), Arable field-minimum tillage (n=9), Arable field-no-till (n=9), Strawberry-organic (n=9), Strawberry-IPM (n=9), Orchard-organic (n=9), and Orchard-IPM (n=9). Four modifications of the QBS index were calculated to estimate soil degradation. QBS methodology foresees computation of one QBS value based on EMI

values of organisms collected in three samples. Therefore, for each system-method group, three QBS index values were obtained out of nine samples. QBS index values as a proxy for soil health were compared between the seven system-method groups using ANOVA with Tukey post-hoc tests.

Results

Examination of the cumulative abundance of taxonomic groups showed *Acarina* were the dominant group in all three arable field management systems, but their dominance was less pronounced in orchard and strawberry fields (Figure 2 A). Log transformation of the abundance improved visualization and statistics for the less abundant groups. The more pronounced differences were for *Hymenoptera* (ants), which were more abundant in the orchard but not in other systems, and larvae of *Coleoptera* and *Diptera*, which were more abundant in arable fields but very few were found in other systems (Figure 2 B).

Overall, 63 soil samples from three agroecosystems and 56.6 % (30) soil-adapted eco-morphological groups were recorded from a total of 53 as defined by the QBS index classification of mesofauna. In orchards, rarefaction curves of morpho-taxonomic groups showed sufficient sampling effort, but for arable and strawberry fields, the rarefaction curves did not reach an asymptote (Figure 3). Considering the rarefaction curve slope for the last five samples, we would gain approximately 0.35 and 0.25 additional eco-morphologic groups with each sample in strawberry and arable fields, respectively (Figure 3 A). As QBS methodology foresees the collection of three samples per sampling site we analysed the richness of eco-morphologic groups in the first three samples of each system-method groups. According to the confidence intervals of these rarefaction curves, the first three samples would detect between 30.5 and 78.0 percent of eco-morphologic groups (Figure 3 B).

PCA analysis was used to visualize differences in the community composition of mesofauna (based on eco-morphologic QBS index groups) for all three sampled agricultural ecosystems, with the first axis explaining 15.8 % of the variability and the second axis explaining 14.7 % of the variability (Figure 4). The first PCA axis was mostly determined by the abundance of very numerous *Collembola* (Figure 4 B) and showed large variability in the community structure of arable fields, especially among minimum tillage samples and lower variability in strawberry field and orchard samples. The seven system-method groups differed significantly ($F=5.3$, $df=6$, $p<0.001$), with post-hoc tests indicating statistically higher values of PCA scores in arable field under minimum tillage compared to arable conventional tillage and all orchard and strawberry field samples under both organic and IPM management. The average species score for the three groups (epiedaphic, hemiedaphic, euedaphic) had a similar length and direction (Figure 4 A).

The second PCA axis separated samples according to less abundant groups such as *Isopoda*, *Diplura*, *Coleoptera* and *Symphyla* (Figure 4 B). PCA sample scores on the second axis were less variable with no statistical differences between the seven system-method groups ($F=1.8$, $df=6$, $p=0.106$), with one of the orchard samples as a clear outlier (Figure 4 A).

In different systems, we observed between 16 (IPM orchard) and 21 (organic orchard) eco-morphologic QBS groups of soil mesofauna (Table 1).

The results of the QBS index varied considerably between different modifications of the index. According to both QBS-ar and QBS-ar BF, which do not consider the abundance of organisms, the highest values were observed for both the organic strawberry field (Strawberry - eco) and the integrated pest management (IPM) strawberry field (Strawberry - int), as well as the organic orchard, with the lowest values detected in the arable field and IPM orchard (Figure 5). Differences between the seven system-method groups were only marginally significant for QBS-ar ($F=3.2$, $df=6$, $p=0.034$), with no significant difference detected by post-hoc tests, and were not significant for QBS-ar BF ($F=2.1$, $df=6$, $p=0.122$).

On the contrary, the two modifications of the QBS index, which consider the abundance of eco-morphological groups, showed higher values for arable fields compared to fruit production agroecosystems (Figure 5). According to the QBS-ab system method, groups differed significantly ($F=8.5$, $df=6$, $p<0.001$), with the post-hoc test indicating higher values for all arable samples compared to strawberry fields under both organic and IPM management (Figure 5). Post-hoc tests also detected significantly higher values in arable minimum tillage compared to both organic and IPM orchard. The QBS-a also differed significantly between the agroecosystems ($F=10.2$, $df=6$, $p<0.001$), where arable minimum tillage received significantly higher QBS-a values than both fruit production systems under organic and IPM management (Figure 5). Furthermore, the QBS-a index of the arable field under minimum tillage was also significantly higher than the conventionally tilled arable field (Figure 5).

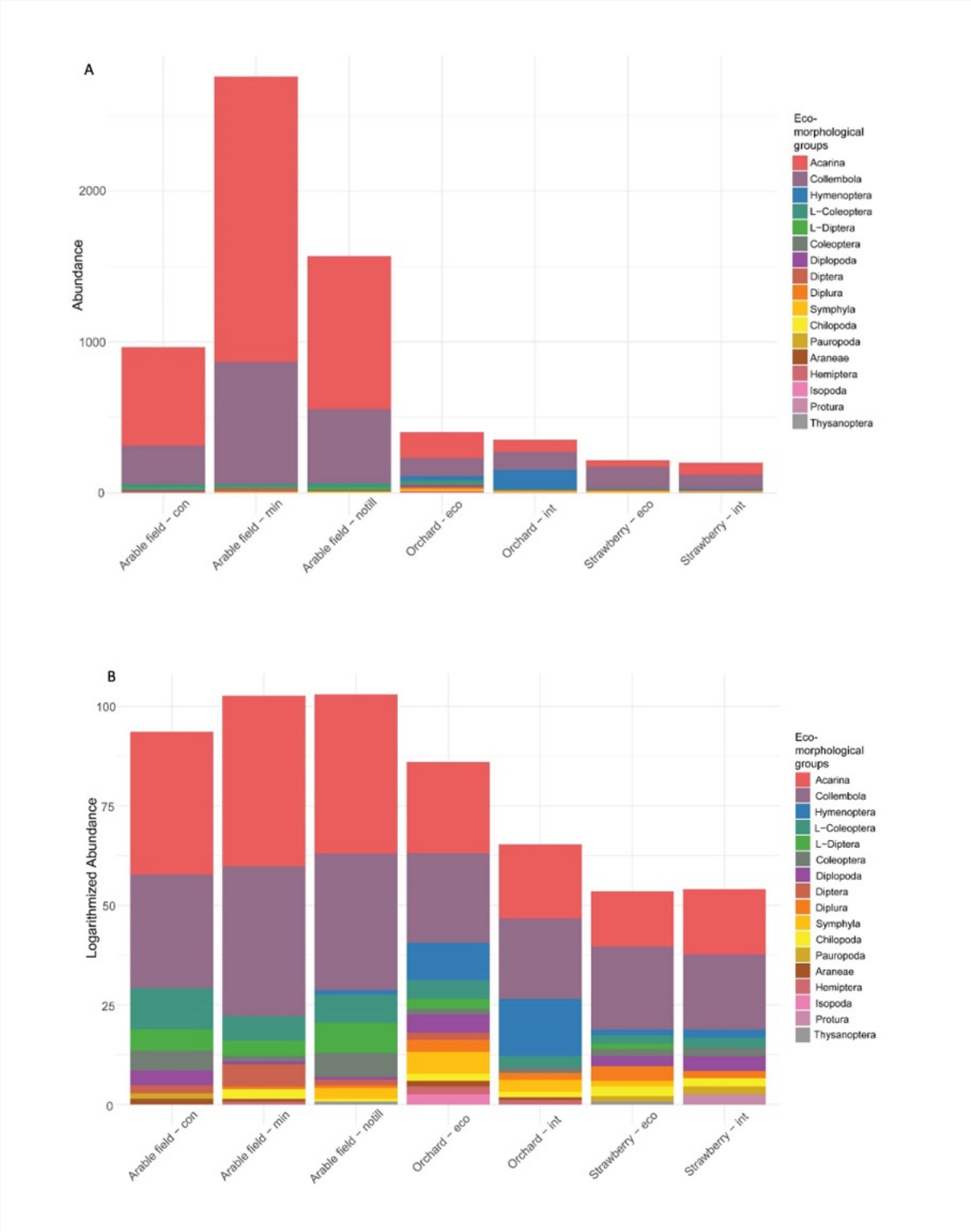

Figure 2. Cumulative abundance (A) and log-transformed cumulative abundance (B) of 17 taxonomic groups of soil mesofauna in arable field, orchard and strawberry agroecosystems of Slovenia with different management regimes. L indicates larval stages.

Slika 2. Kumulativna številčnost (A) in logaritmično transformirana kumulativna številčnost (B) 17 taksonomskih skupin talne mezofavne v njivi, sadovnjaku in nasadu jagod z različnimi režimi upravljanja. L označuje larvalne stadije.

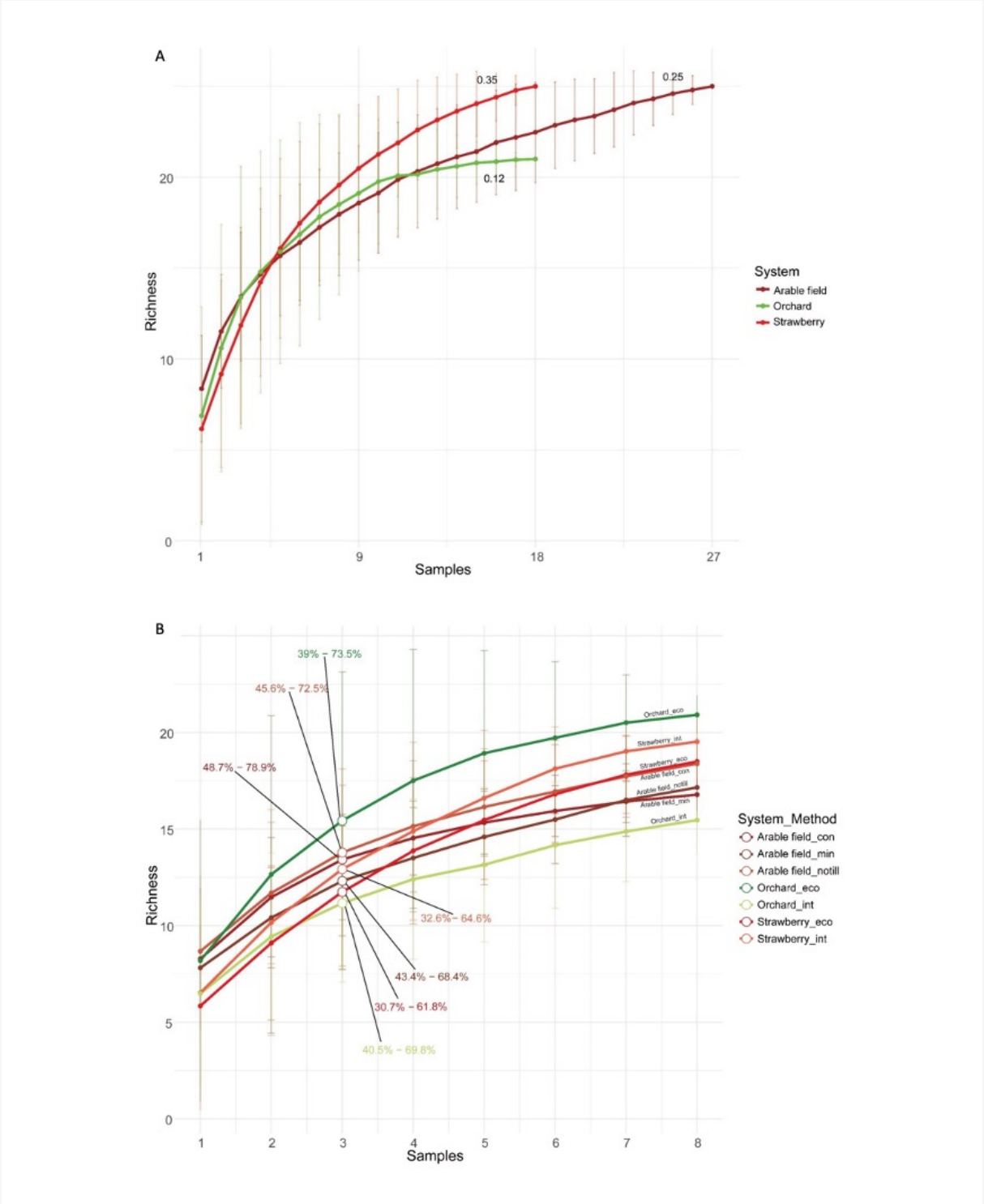

Figure 3. Rarefaction curves with 95% confidence intervals of morpho-taxonomic groups of soil mesofauna according to QBS methodology in A) three agroecosystems with numbers indicating slope for the last five samples and B) seven system-method groups with numbers indicating the percentage of morpho-taxonomic groups detected in the first three samples per sampling site, as required by the QBS methodology.

Slika 3. Rarefakcijske krivulje s 95 % intervali zaupanja za morfo-taksonomske skupine talne mezofavne po metodologiji QBS v A) treh agroekosistemih s številkami, ki prikazujejo naklon za zadnjih pet vzorcev, in B) sedmih sistemsko-metodoloških skupinah s številkami, ki označujejo odstotek morfo-taksonomskih skupin, zaznanih v prvih treh vzorcih na vzorčnem mestu, kot zahteva metodologija QBS.

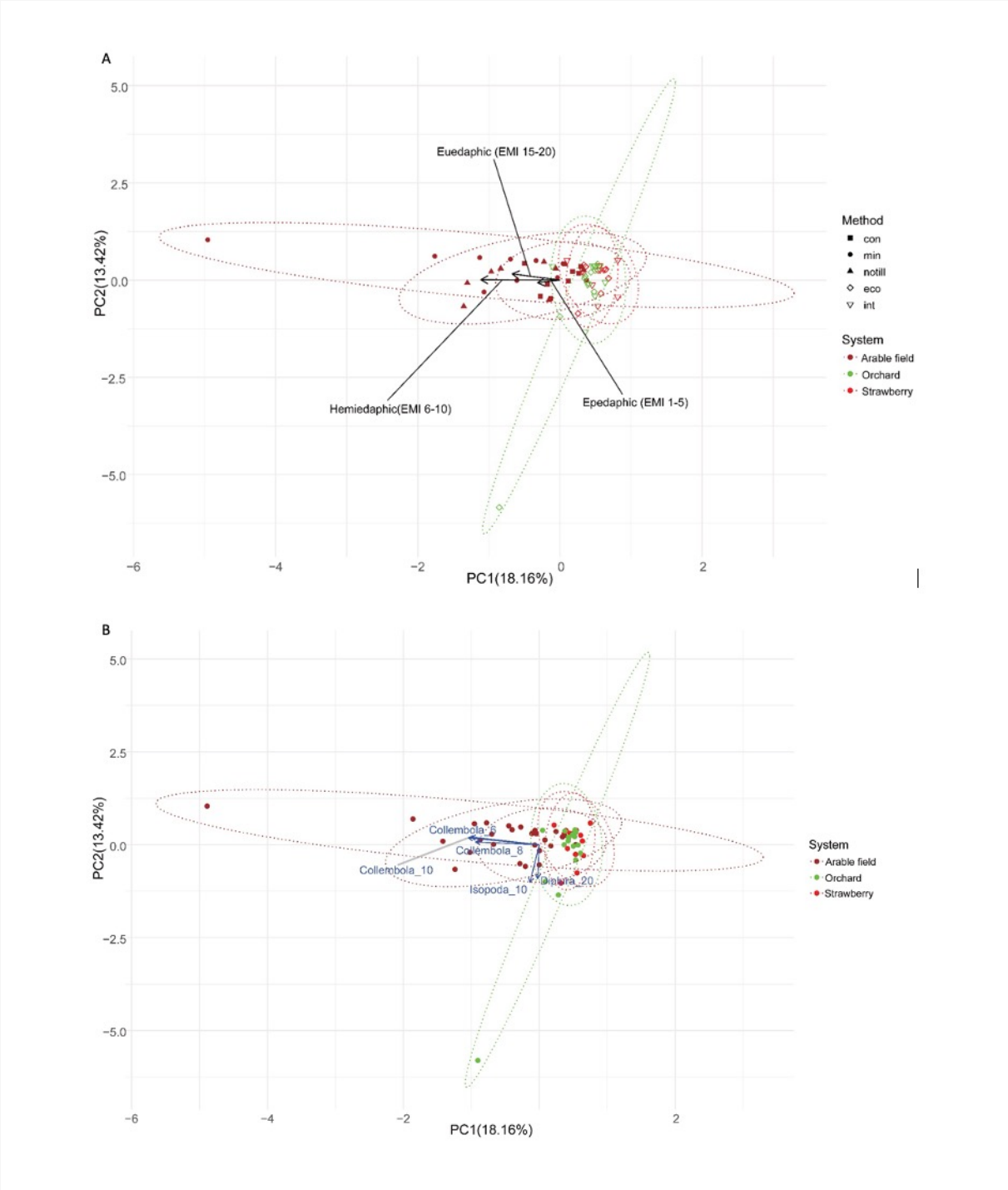

Figure 4. Principal component analysis (PCA) scores on the first two axes of A) arable field, strawberry field, and orchard under different management systems (int = IPM; eco = organic; con = conventional tillage; min = minimum tillage; notill = no tillage) with arrows indicating average species scores of epiedaphic, hemiedaphic and euedaphic groups and B) arable, strawberry field and orchard samples with arrows indicating species scores of 5 eco-morphologic groups with the highest species scores on both axes.

Slika 4. Rezultati analize glavnih komponent (PCA) na prvih dveh oseh za A) njivo, nasad jagod in sadovnjak pod različnimi režimi upravljanja (int = integrirano varstvo rastlin; eco = ekološko kmetovanje; con = konvencionalna obdelava tal; min = minimalna obdelava tal; notill = brez obdelave tal) s puščicami, ki kažejo povprečne rezultate vrst epiedafičnih, hemiedafičnih in euedafičnih skupin, ter B) vzorce njive, nasada jagod in sadovnjaka s puščicami, ki označujejo rezultate vrst za 5 ekomorfoloških skupin z najvišjimi rezultati vrst na obeh oseh.

Table 1. Number of detected eco-morphological groups for soil samples collected in three agroecosystems under different management.

Tabela 1. Število zaznanih ekomorfoloških skupin za vzorce tal, zbrane v treh agroekosistemih pod različnimi režimi upravljanja.

Agricultural ecosystem	Management	Number of eco-morphological groups
Arable field	Conventional tillage	17
Arable field	Minimum tillage	18
Arable field	No-till	19
Strawberry field	Organic	19
Strawberry field	IPM	20
Orchard	Organic	21
Orchard	IPM	16

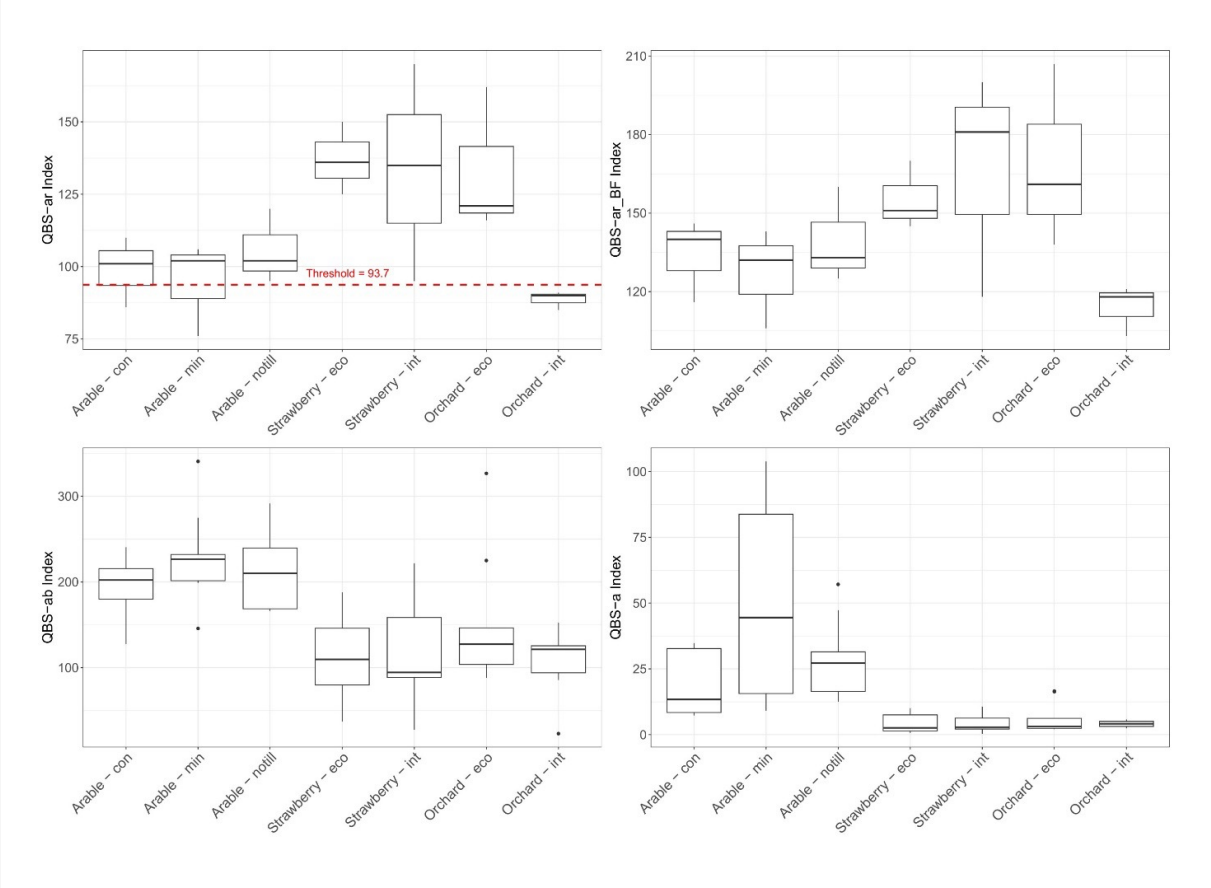

Figure 5. Median and quartile values of four QBS modifications: two without considering animal abundances (QBS-ar, QBS-ar BF), and two considering abundances (QBS-ab and QBS-a) for soil samples from seven system-method groups. The red line indicates a tentative threshold value of 93.7 for the QBS-ar index, separating high-quality soils above the threshold from poor-quality soils.

Slika 5. Mediana in kvartilne vrednosti štirih različic QBS za talne vzorce iz sedmih sistem-metoda skupin. Dva ne upoštevata abundance živali (QBS-ar in QBS-ar BF), dva pa abundanco upoštevata (QBS-ab in QBS-a). Rdeča črta predstavlja predlagano mejno vrednost QBS-ar (93,7) nad katero so tla visoke kakovosti, pod pa tla nizke kakovosti.

Discussion

Across all studied sites, *Acarina* and *Collembola* were the most dominant in samples. These two groups are the two most abundant and diverse taxa of soil mesofauna, which have also been the most investigated (Menta & Remelli, 2020). Their dominance in our samples was unsurprising as it has been reported in similar studies in Europe with the proportion of *Acarina* between 48-57 % and *Collembola* around 30 % in vineyards (Gagnarli et al., 2015), with similar proportions in olive orchards (Vignozzi et al., 2019). Similar proportions with dominant *Acarina* (65 %) and fewer *Collembola* (27.6 %) were reported in forest sites. However, these proportions were inverted in favour of *Collembola* (56.7 %) compared to *Acarina* (39.8 %) in cropland and meadows in a systematic study in France (Cluzeau et al., 2012).

In our study, the dominance of *Acarina* over *Collembola* was much more pronounced in arable fields under all three tillage methods but less pronounced in strawberry fields and orchards. This reduced abundance of *Acarina* in the strawberry field and orchard could be due to the higher sensitivity of *Acarina* to soil contaminants such as heavy metals compared to *Collembola* (Menta et al., 2008), although some experimental studies contradict this (Joimel et al., 2017). Considering the heavy use of copper-based pesticides in fruit production, the lower relative abundance of *Acarina* compared to *Collembola* in orchard and strawberry production could indicate their sensitivity to soil contaminants, although further chemical analysis of soil would be needed to confirm this. Additionally, the presence of black polyethene mulch in both the organic and IPM sections of the strawberry plantation at the Infrastructure Center Brdo may have contributed to a background level of microplastics in these soils. While no specific analysis of microplastics was conducted in this study, it is worth noting that microplastics are an emerging concern for soil health and may influence mesofaunal communities (Jemec Kokalj et al., 2024). Studies indicate that microplastics can adversely affect soil biodiversity and the health of mesofauna, including groups such as *Acarina* and *Collembola* (Shafea et al., 2023). Although assessing microplastic impacts was beyond the scope of this study, future research should consider this factor, particularly in systems where plastic mulching is commonly used.

Collembola, on the other hand, responds more to the soil perturbation associated with agricultural practices such as tillage and fertilization (Cluzeau et al., 2012). Reduced tillage had a positive effect on *Collembola* abundance compared to conventional tillage, with effect varying due to depth, climate, soil texture, but also tillage method and frequency and concurrent herbicide application (Betancur-Corredor et al., 2022). The positive effect of reduced tillage on *Collembola* abundance was also confirmed in our study of arable fields using different tillage methods.

PCA analysis showed that tillage intensity had a much greater effect on soil mesofauna community in arable fields than did the type of agroecosystem or the production method (organic or IPM). Variability in the abundance of different eco-morphological groups of *Collembola* seemed to be the most important driver of discrimination between our samples, as indicated by the first PCA axis. This is in line with the results of Chassain et al. (2024), who examined the effects of cropping systems on soil mesofauna density and diversity in 21 fields using practice intensity indicators and indexes and found that the tillage intensity index showed a major impact. In the case of our tested sites, the differences between organic and IPM fruit production varied in more than one management practice, and it is consequently impossible to disentangle their individual effects on soil biota. In the case of the orchard, the two methods differ in the types of pesticides used on trees (synthetic pesticides in IPM and copper-based pesticides in organic), in types of fertilizer (mineral fertilizers in IPM and organic fertilizers in organic) and in the application of herbicides for weed suppression under trees (IPM) and mechanical disturbance for weed removal (organic). In their recent review of below-ground arthropod diversity in conventional and organic vineyards (Di Giovanni et al., 2024), they pointed to unclear management aspects of organic versus conventional farming as the reasons for conflicting responses in soil biota. The same authors also stressed the importance of assessing individual management practices for soil biota functional groups.

The QBS-ar index values in our analysis (76–170) were comparable to those reported in other agroecosystem studies (Menta et al., 2018). A tentative threshold value of 93.7 was proposed for QBS-ar to distinguish high-quality soils from poorer ones (Menta et al., 2018). According to this criterion, all sampled management groups except the IPM orchard could be categorised as good-quality soils, with the strawberry field having the highest values. However, the QBS-ar index was not able to distinguish between the different tillage systems due to the high variability, resulting in no statistically significant differences

between the farming systems. This suggests that while the QBS-ar index is sufficient for distinguishing environments with very different practices, it is not sensitive enough to detect subtle changes resulting from specific management practices such as tillage. Interestingly, the strawberry field — a highly disturbed environment covered with plastic mulch had high QBS-ar values. This could be due to the low number of organisms in the samples, which made it easier for the analyst to identify new ecomorphological groups (EMI) and thus increase the overall score. This highlights a potential limitation of the QBS-ar index: it may overestimate soil quality in disturbed environments where, due to sampling artefacts, low organism diversity coincides with an apparently high diversity of EMI. Conversely, environments with a high number of mesofauna — possibly due to high input of organic matter in disturbed environments — do not necessarily reflect better soil health. High numbers of mesofauna feeding on introduced organic matter may be a response to disturbance rather than a sign of a healthy, stable ecosystem. When looking at organism abundance, the QBS results were reversed, with arable areas with different tillage methods showing the highest values. Only the indices that included abundance (QBS-ab and QBS-a) were able to distinguish between different tillage practices, which is consistent with the PCA analysis. These indices, which are sensitive to changes in population density due to agricultural interventions such as tillage, were indeed able to detect abundance-related differences. These results emphasise the complexity of assessing soil health using these indices. Non-abundance indices (QBS-ar) provide information on overall soil quality and ecological stability but may not accurately reflect the nuances of disturbed environments or the effects of organism abundance. Indices with abundance (QBS-ab and QBS-a) provide a more detailed insight into the effects of management actions on soil mesofauna but do not necessarily correlate with improved soil health. Our study emphasises the need for more comprehensive methods to assess soil biodiversity and health. Although QBS indices provide valuable information, they can only capture the dynamics of the soil ecosystem to a limited extent. New techniques such as DNA metabarcoding could provide deeper insights by enabling more accurate identification and quantification of soil organisms and thus improve our understanding of soil health in different agroecosystems. We recommend that researchers carefully consider their objectives and the limitations of each index when selecting a soil biodiversity assessment method. For general assessments of soil quality or ecological stability in very different environments, the QBS-ar may be appropriate but should be used with caution in disturbed environments with low organismal diversity. To detect subtle changes and examine the effects of agricultural practices where organism diversity varies, QBS-ab and QBS-a are more suitable, although they may not fully reflect soil health. Ultimately, a multifaceted approach that combines traditional indices with advanced molecular techniques may be necessary to gain a comprehensive understanding of soil biodiversity and health. This integrated strategy would contribute to better-informed agricultural management and conservation efforts by enabling a more accurate assessment of the impact of different practices on soil ecosystems.

Originally, the QBS methodology foresees the collection of three samples per sampling site, but we increased the effort to nine samples. Rarefaction curves showed that nine samples were sufficient for orchards but not for arable and strawberry fields, where the asymptote was not reached. According to the rarefaction curves, only between 30.5 and 78 percent of eco-morphologic groups would be detected by collecting three samples. This indicates that spatial heterogeneity of soil mesofauna differs between the agroecosystems, and the number of samples should be higher than the three originally proposed in more heterogeneous ecosystems.

Although this study was limited to three agroecosystems with only one sampling site, it gives a valuable first insight into the diversity of soil mesofauna within and between agroecosystems. A comparison of different tillage and production methods identified tillage as an important factor determining the soil mesofauna community. However, the complexity of agricultural practices in organic and IPM fruit production makes it impossible to disentangle the environmental factors affecting this community.

Supplementary Materials

Suppl. Table S1. Abundances of collected taxonomic groups of mesofauna and their proportion of the total sample.

Author Contributions

Conceptualization, V.N, I.B., N.Š., T.G.; methodology, V.N., I.B., N.Š..; formal analysis, V.N.; writing—original draft preparation, V.N. and I.B.; writing—review and editing, N.Š. and T.G.; visualization, V.N.; All authors have read and agreed to the published version of the manuscript.

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Data Availability

Data supporting this study will be made available upon request to the corresponding author.

Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

ARSO. 2023. [Dataset]. https://meteo.arso.gov.si/met/sl/climate/maps/

Basset, Y., Hajibabaei, M., Wright, M. T. G., Castillo, A. M., Donoso, D. A., Segar, S. T., Souto-Vilarós, D., Soliman, D. Y., Roslin, T., Smith, M. A., Lamarre, G. P. A., De León, L. F., Decaëns, T., Palacios-Vargas, J. G., Castaño-Meneses, G., Scheffrahn, R. H., Rivera, M., Perez, F., Bobadilla, R., … Barrios, H., 2022. Comparison of traditional and DNA metabarcoding samples for monitoring tropical soil arthropods (Formicidae, Collembola and Isoptera). Scientific Reports, 12(1), 10762. https://doi.org/10.1038/s41598-022-14915-2

Behan-Pelletier, V. M., 2003. Acari and Collembola biodiversity in Canadian agricultural soils. Canadian Journal of Soil Science, 83(Special Issue), 279–288. https://doi.org/10.4141/S01-063

Betancur-Corredor, B., Lang, B., Russell, D. J., 2022. Reducing tillage intensity benefits the soil micro- and mesofauna in a global meta- analysis. European Journal of Soil Science, 73(6), e13321. https://doi.org/10.1111/ejss.13321

Chassain, J., Joimel, S., Gardarin, A., Gonod, L. V., 2024. Indicators of practice intensity unearth the effects of cropping systems on soil mesofauna. Agriculture, Ecosystems & Environment, 362, 108854. https://doi.org/10.1016/j.agee.2023.108854

Cluzeau, D., Guernion, M., Chaussod, R., Martin-Laurent, F., Villenave, C., Cortet, J., Ruiz-Camacho, N., Pernin, C., Mateille, T., Philippot, L., Bellido, A., Rougé, L., Arrouays, D., Bispo, A., Pérès, G., 2012. Integration of biodiversity in soil quality monitoring: Baselines for microbial and soil fauna parameters for different land-use types. European Journal of Soil Biology, 49, 63–72. https://doi.org/10.1016/j.ejsobi.2011.11.003

D'Avino, L., Bigiotti, G., Vitali, F., 2023. QBS-ar and QBS-ar_BF index toolbox for biodiversity assessment of microarthropods community in soil. Zenodo. https://doi.org/10.5281/zenodo.7778672

Di Giovanni, F., Nardi, F., Frati, F., Migliorini, M., 2024. Below-ground arthropod diversity in conventional and organic vineyards: A review. Crop Protection, 180, 106666. https://doi.org/10.1016/j.cropro.2024.106666

Foucher, A., Evrard, O., Ficetola, G. F., Gielly, L., Poulain, J., Giguet-Covex, C., Laceby, J. P., Salvador-Blanes, S., Cerdan, O., Poulenard, J., 2020. Persistence of environmental DNA in cultivated soils: Implication of this memory effect for reconstructing the dynamics of land use and cover changes. Scientific Reports, 10(1), 10502. https://doi.org/10.1038/s41598-020-67452-1

Gagnarli, E., Goggioli, D., Tarchi, F., Guidi, S., Nannelli, R., Vignozzi, N., Valboa, G., Lottero, M. R., Corino, L., Simoni, S., 2015. Case study of microarthropod communities to assess soil quality in different managed vineyards. SOIL, 1(2), 527–536. https://doi.org/10.5194/soil-1-527-2015

General Secretariat of the Council, 2024. Proposal for a Directive of the European Parliament and of the Council on Soil Monitoring and Resilience (Soil Monitoring Law) (No. 11299/24). Council of the European Union. https://eur-lex.europa.eu/

Guerra, C. A., Heintz-Buschart, A., Sikorski, J., Chatzinotas, A., Guerrero-Ramírez, N., Cesarz, S., Beaumelle, L., Rillig, M. C., Maestre, F. T., Delgado-Baquerizo, M., Buscot, F., Overmann, J., Patoine, G., Phillips, H. R. P., Winter, M., Wubet, T., Küsel, K., Bardgett, R. D., Cameron, E. K., … Eisenhauer, N., 2020. Blind spots in global soil biodiversity and ecosystem function research. Nature Communications, 11(1), 3870. https://doi.org/10.1038/s41467-020-17688-2

Hornsby, A. G., Herner, A. E., Don Wauchope, R., 1996. Pesticide Properties in the Environment. Springer. https://doi.org/10.1007/978-1-4612-2316-0

Jemec Kokalj, A., Nagode, A., Drobne, D., Dolar, A., 2024. Effects of agricultural microplastics in multigenerational tests with insects; mealworms Tenebrio molitor. Science of The Total Environment, 946, 174490. https://doi.org/10.1016/j.scitotenv.2024.174490

Jin, Q., Han, H., Hu, X., Li, X., Zhu, C., Ho, S. Y. W., Ward, R. D., Zhang, A., 2013. Quantifying Species Diversity with a DNA Barcoding-Based Method: Tibetan Moth Species (Noctuidae) on the Qinghai-Tibetan Plateau. PLoS ONE, 8(5), e64428. https://doi.org/10.1371/journal.pone.0064428

Joimel, S., Schwartz, C., Hedde, M., Kiyota, S., Krogh, P. H., Nahmani, J., Pérès, G., Vergnes, A., Cortet, J., 2017. Urban and industrial land uses have a higher soil biological quality than expected from physicochemical quality. Science of The Total Environment, 584–585, 614–621. https://doi.org/10.1016/j.scitotenv.2017.01.086

Kempson, D. L., 1963. A new extractor for woodland litter. Pedobiologia, 3, 1–21.

Lešnik, M., Tojnko, S., Solar, A., Usenik, V., Koron, D., Turinek, Matjaž., Godec, B., Vrhovnik, I., Jančar, M., Brence, A., Bajec, D., Rodič, K., Caf, A., 2016. Tehnološka navodila za ekološko pridelavo sadja. Ministrstvo za kmetijstvo, gozdarstvo in prehrano.

Mantoni, C., Pellegrini, M., Dapporto, L., Del Gallo, M. M., Pace, L., Silveri, D., Fattorini, S., 2021. Comparison of Soil Biology Quality in Organically and Conventionally Managed Agro-Ecosystems Using Microarthropods. Agriculture, 11(10), Article 10. https://doi.org/10.3390/agriculture11101022

Menta, C., Conti, F. D., Pinto, S., 2018. Microarthropods biodiversity in natural, seminatural and cultivated soils—QBS-ar approach. Applied Soil Ecology, 123, 740–743. https://doi.org/10.1016/j.apsoil.2017.05.020

Menta, C., Leoni, A., Bardini, M., Gardi, C., Gatti, F., 2008. Nematode and Microarthropod Communities: Comparative Use of Soil Quality Bioindicators in Covered Dump and Natural Soils. Environmental Bioindicators, 3(1), 35–46. https://doi.org/10.1080/15555270701885762

Menta, C., Remelli, S., 2020. Soil Health and Arthropods: From Complex System to Worthwhile Investigation. Insects, 11(1), 54. https://doi.org/10.3390/insects11010054

Ogrin, D., Repe, B., Štaut, L., Svetlin, D., Ogrin, M., 2023. Podnebna tipizacija Slovenije po podatkih za obdobje 1991–2020. Dela, 59, 5–89. https://doi.org/10.4312/dela.59.5-89

Orgiazzi, A., Dunbar, M. B., Panagos, P., De Groot, G. A., Lemanceau, P., 2015. Soil biodiversity and DNA barcodes: Opportunities and challenges. Soil Biology and Biochemistry, 80, 244–250. https://doi.org/10.1016/j.soilbio.2014.10.014

Parisi, V., Menta, C., Gardi, C., Jacomini, C., Mozzanica, E., 2005. Microarthropod communities as a tool to assess soil quality and biodiversity: A new approach in Italy. Agriculture, Ecosystems & Environment, 105(1–2), 323–333. https://doi.org/10.1016/j.agee.2004.02.002

Pravilnik o ekološki pridelavi in predelavi kmetijskih pridelkov oziroma živil, 2018. Version Uradni list RS, 72/18, 17/19 – popr., 105/22, 2018.

Pravilnik o integrirani pridelavi poljščin, zelenjave, hmelja, sadja in oljk ter grozdja, 2023. Version Uradni list RS, št. 31/23, 67/23, 2023.

Shafea, L., Yap, J., Beriot, N., Felde, V. J. M. N. L., Okoffo, E. D., Enyoh, C. E., Peth, S., 2023. Microplastics in agroecosystems: A review of effects on soil biota and key soil functions. Journal of Plant Nutrition and Soil Science, 186(1), 5–22. https://doi.org/10.1002/jpln.202200136

Tabaglio, V., Gavazzi, C., Menta, C., 2009. Physico-chemical indicators and microarthropod communities as influenced by no-till, conventional tillage and nitrogen fertilisation after four years of continuous maize. Soil and Tillage Research, 105(1), 135–142. https://doi.org/10.1016/j.still.2009.06.006

Vignozzi, N., Agnelli, A. E., Brandi, G., Gagnarli, E., Goggioli, D., Lagomarsino, A., Pellegrini, S., Simoncini, S., Simoni, S., Valboa, G., Caruso, G., Gucci, R., 2019. Soil ecosystem functions in a high-density olive orchard managed by different soil conservation practices. Applied Soil Ecology, 134, 64–76. https://doi.org/10.1016/j.apsoil.2018.10.014

Review

Designer cellulosomes – catalytic nanomachines with significant potential in biotechnology and circular economy

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Abstract

Cellulosomes are multienzyme complexes originally found on the surface of certain anaerobic cellulolytic bacteria and fungi specialized in the degradation of plant cell walls. Recently, the efficiency in lignocellulose conversion and architectural features of these intricate complexes inspired the construction of artificial chimeric complexes for targeted substrate degradation. The simultaneous advancements in synthetic biology, protein engineering and the pursuit of greater sustainability across various industries have highlighted the immense potential of these artificially designed enzymatic complexes for diverse applications. Notably, they hold significant promise for industries specializing in the valorization of plant biomass waste and the production of bio-based renewable energy. The article discusses the main architectural features, design and construction steps, and various biotechnological applications of these intriguing nanomachines.

Keywords

Designer cellulosomes; lignocellulose valorization; protein engineering; nanobiotechnology, bio-based products

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Sintetični celulosomi – katalitični nanostroji z velikim potencialom v biotehnologiji in krožnem gospodarstvu

Izvleček

Celulosomi so multiencimski kompleksi, primarno identificirani na površini nekaterih anaerobnih celulolitičnih bakterij in gliv specializiranih za razgradnjo rastlinskih celičnih sten. Zaradi izjemne učinkovitosti pri razgradnji odpornih lignoceluloznih materialov ter njihovih edinstvenih arhitekturnih značilnosti so postali navdih za razvoj sintetičnih hibridnih analogov zasnovanih za ciljno razgradnjo različnih (predvsem odpadnih) lignoceluloznih substratov. Hiter razvoj sintezne biologije, napredki v proteinskem inženirstvu ter vse večja potreba po trajnostnih rešitvah obstoječih izzivov so vodili do spoznanja, da imajo ti umetno zasnovani katalitični nanostroji velik potencial v mnogih industrijskih panogah, ki slonijo na učinkoviti razgradnji lignocellulozne biomase. Še posebno se pričakuje, da bi lahko ti umetni kompleksi pomembno doprinesli v industrijskih aplikacijah, ki temeljijo na valorizaciji odpadne rastlinske biomase, zlasti v sektorjih osredotočenih na pridobivanje obnovljive energije in bele biotehnologije. Članek se osredotoča na glavne arhitekturne značilnosti, zasnovo in ključne korake pri konstrukciji sintetičnih celulosomov ter njihov potencial za različne biotehnološke aplikacije.

Ključne besede

celulosomi; valorizacija lignoceluloze; proteinski inženiring, sintezna biologija, nanobiotehnologija, bio-osnovani produkti

Introduction

As the world strives for alternatives to fossil fuels and more sustainable methods for managing agricultural and industrial waste, leveraging the evolutionarily refined natural systems capable of efficiently degrading accumulating plant biomass is becoming increasingly important (Gayathri et al., 2021). Among the most elaborated systems capable of efficient degradation of lignocellulose have been found on the surface of some anaerobic microorganisms. Cellulosomes are large, multi-enzyme complexes primarily produced by anaerobic cellulolytic bacteria, particularly from the order *Clostridiales*, inhabiting different environments rich in lignocellulosic materials, such as soil, compost and rumen (Bayer et al., 2004). The pioneering work that established the cellulosome paradigm began over four decades ago with Bayer and Lamed (Bayer et al., 1983), who first described the extracellular cellulolytic complex of *Clostridium thermocellum*. Subsequent research uncovered similar or even more intricate complexes in other anaerobic (hemi)cellulolytic bacteria, with the most extensively characterized examples being the cellulosomes of *Clostridium cellulolyticum, Clostridium cellulovorans, Acetivibrio cellulolyticus*, and *Ruminococcus flavefaciens* and fungi (Artzi et al., 2017). Recently, a minimalistic cellulosome has also been described in the butanologenic bacterium *Clostridium saccharoperbutylacetonicum* (Levi Hevroni et al., 2024). Cellulosomes in these species are specialized for degrading the recalcitrant plant cell wall polysaccharides, including cellulose, hemicellulose, and pectin. Their exceptional efficiency in breaking down lignocellulosic biomass is due to their highly organized structure and synergistic action of multiple enzymes positioned close to one another (Alves et al., 2020). With the recent rise of white and green biotechnology followed by circular economy movements, these efficient cellulolytic complexes attracted significant attention as potential catalysts for the valorization of lignocellulose waste, not only because of their catalytic efficiency but also due to their specific architectural features. The modular architecture of structural and catalytic subunits composing these complexes was recognized as potential building blocks for the construction of designer catalytic complexes that can be adapted for specific types of plant biomass (feedstocks), potentially expressed in different hosts and used in various industrial applications (Asemoloye et al., 2023).

Cellulosome architecture

The unique architecture of cellulosomes is critical to their function. The primary components of each cellulosome include one or more structural proteins, scaffoldins, and several catalytic subunits. Primary scaffoldin acts as the backbone of the cellulosome architecture, anchoring the complex to the substrate on the one hand and providing firm docking sites for enzymatic subunits on the other. The enzymatic machinery of cellulosomes comprises a diverse set of carbohydrate-active enzymes (CAZYmes) involved in the degradation of plant cell walls, particularly glycoside hydrolases, carbohydrate esterases, and polysaccharide lyases (Artzi et al., 2017). Furthermore, proteases and their inhibitors (SERPINS) have also been found within some complexes. The spatial organization of the enzymes within the complex minimizes diffusion limitations, enhancing the overall efficiency of the complex (Alves et al., 2021).

The main interaction holding together the subunits in cellulosome is the high-affinity interaction between cohesin modules on the scaffoldins and dockerin modules on the enzyme subunits (Type I cohesin-dockerin interaction) (as depicted in Fig. 1). This interaction allows dynamic assembly of cellulosome components and adaptation to the enzyme repertoire based on substrate availability. In addition, a different type of cohesin is also present on the secondary, anchoring scaffoldin (Type II), known to bind the main scaffoldin on one side and attach to the bacterial cell wall from the other (Artzi et al., 2017). Some cellulosomes, for example, the complexes identified in *R. flavefaciens*, are known for even more elaborate structures, involving several scaffoldins binding with each other, resulting in multiplied enzyme-binding sites (Vodovnik et al., 2013; Stern et al., 2016). Furthermore, adaptor scaffoldins have also been discovered that enable switching the substrate specificity of the complex by accommodating different sets of the synthesized enzymes.

Most of the scaffoldins (and some enzyme polypeptides) also contain a carbohydrate-binding module (CBM), which plays a crucial role in targeting and binding to specific carbohydrates, enhancing the catalytic efficiency of the complex by bringing the enzymes in close proximity to the substrate. According to structural properties, CBMs are classified in different families. Some CBM families (for example, CBM 1,2 and 3) target crystalline or amorphous cellulose through hydrophobic stacking interaction and hydrogen bonding with glucose residues. Others, such as CBM families 6 and 22, bind to hemicellulose components like xylan, mannan or arabinoxylan. Their specificity depends on the sugar composition of the hemicellulose substrate. In addition, pectin-binding CBMs targeting homogalacturonan or rhamnogalacturonan (CBM family 28) have also been identified. Typically, the main scaffoldin identified within the cellulosomal scaffoldins belongs to the family CBM3a, which is known to recognize regular repeating structures on insoluble carbohydrate surfaces, such as crystalline cellulose or chitin. The catalytic (GH) and structural (CBM, dockers, cohesins) modules in cellulosomes are connected by linker regions, which provide flexibility and enable dynamic movement on the surface of the substrate (Alves et al., 2021).

The attachment of cellulosomes to the bacterial cell walls is another critical aspect of their function in biomass degradation. Several strategies are used to anchor scaffoldins to the bacterial cell wall, including noncovalent interaction via S-layer homology (SLH) domains hydrophobic anchoring to the lipid bilayer, or sortase-mediated covalent anchoring of the scaffoldins to peptidoglycan (Artzi et al., 2017).

Designer cellulosomes

Natural cellulosomes, although highly efficient in natural environments, have several constraints that limit their industrial utility, the main three being: (1) non-optimal enzyme composition (the enzyme repertoire of natural cellulosomes is limited to what the host organism naturally expresses), (2) environmental constraints (natural cellulosomes are often adapted to specific environmental conditions, such as strict anaerobiosis, which may not align with industrial requirements), (3) lack of flexibility (the fixed enzyme arrangement in natural cellulosomes restricts their adaptability to various substrates) (Lamote et al., 2023). These challenges have been tacked by the advances in molecular and synthetic biology, which paved the way for the construction of artificial complexes with customized enzyme combinations with improved flexibility, efficiency, specificity and stability in industrial conditions (Joseph et al., 2018). Designer cellulosomes (depicted in Fig. 1) are modular complexes with previously designed architecture and composition constructed using synthetic biology and protein engineering tools (Vazana et al., 2013). The construction of such engineered complexes is achievable by leveraging the species-specificity inherent

in cohesin-dockerin interactions. Apart from the controlled incorporation of target catalytic activities, the engineering of the cellulosomes also allows for the expression and stability optimization of target complexes. The optimization of different subunits can be performed either by rational engineering approach, relying on previous knowledge of the structure-function relationship, or directed evolution, by constructing a library of random mutants followed by rigorous selection based on desired properties of the proteins. Other approaches to increase the stability of designer cellulosomes in industrially-relevant conditions, such as glycosylation, have also been studied (Khan et al., 2020).

Several such designer complexes targeted for different biotechnological applications, particularly focused on valorization of (hemi)cellulose biomass, have already been constructed and displayed improved activity, stability, and adaptability across diverse substrates (Wen et al., 2010; Ponsetto et al., 2024).

Construction of designer cellulosomes

The construction of the designer cellulosomes is a multi-step process that is based on previous knowledge regarding the structure-function relationship of different protein components. The main steps in the construction of designer cellulosomes involve (1) selection of target substrate to be degraded (transformed in value-added products); (2) selection of optimal enzyme activities and stochiometry for optimal degradation of the target substrate (based on its composition, i.e. hemicellulose or cellulose prevalence, crystallinity, branching..); (3) complex design: selection of the modules and design of the cellulosome components (scaffoldin design, including the number and specificity of cohesin domains based on the number and type of enzymes to be incorporated and enzymatic subunits design, involving target catalytic domains and compatible dockerin modules); (4) construction of vectors encoding designed modular proteins, molecular cloning to construct

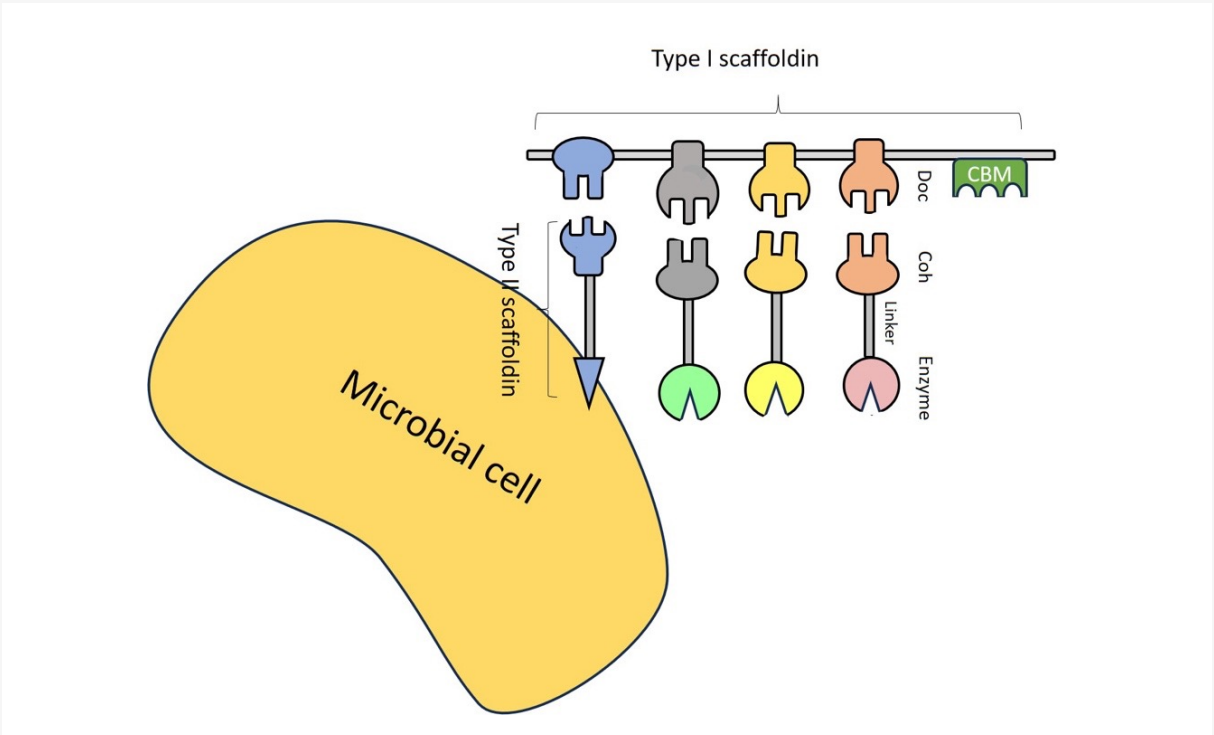

Figure 1. Graphic representation of the basic architecture of a designer cellulosome. Doc: dockerin, Coh: cohesin, CBM: carbohydrate-binding module. Differences in colours represent different sources of protein modules within the complex (i.e. the sequence of each module can originate from different species).

Slika 1. Grafični prikaz osnovne arhitekture načrtovanega celulosoma. Doc: dokerin, Coh: kohezija, CBM: modul za vezavo ogljikovih hidratov. Razlike v barvah predstavljajo različne vire beljakovinskih modulov v kompleksu (tj. zaporedje vsakega modula lahko izvira iz druge vrste).

coding sequences of the complex subunits; (5) expression of vector constructs in heterologous hosts (selected based on targeted application) and assembly of the subunits into the final complex, (6) *in vitro* determination of stability and activity of target designer complexes (Lamote et al., 2023).

The selection of protein modules includes three main aspects, starting with scaffoldin and protein modules selection, which is followed by carbohydrate-active enzymes (CAZYmes) and carbohydrate-binding modules (CBMs) selection (based on the target substrate and the desired degree of degradation). In addition, accessory proteins, such as adaptor scaffoldins, may also be added (Stern et al., 2016). Enzymes with different specificities have already been included in such designer complexes. Typically, at least one endo- and exoglucanase pair is needed to target cellulose-rich biomass (Arfi et al., 2014; Vazana et al., 2010), while different types of enzymes targeting hemicellulases (xylanases, arabinases, mannanases), expansins (Chen et al., 2016) or even lignin-targeting laccases may also be added (Fierobe et al., 2001; Fierobe et al., 2005). Further, the selection of cohesin-dockerin pairs and possible linkers is also performed, which does not depend on the substrate composition. The efficiency of designer cellulosomes is influenced by many parameters, including the selected dockerins and linkers, as well as the docking enzyme ratio on the scaffoldin. Recently, the Versatile platform was successfully applied to construct a range of different docking enzymes and scaffoldin variants, facilitating the targeted study of specific parameters influencing the activity of designer cellulosomes. This platform includes a tile repository composed of dockerins, cohesins, linkers, tags and catalytic modules and allows for the fast and efficient construction of designer cellulosomes, enabling the creation of a practically infinite number of complexes (Vanderstraeten et al., 2022).

After selecting the basic components, the final complex architecture is designed by deciding the order of different modules in scaffoldin(s) and designer enzymes, which considers enzyme synergy and potential spacial constraints (Caspi et al., 2009).

In the next step, modular DNA encoding dockerin-containing enzymes and scaffoldin(s) is either synthesized or constructed by different cloning techniques (restriction-digestion, CPEC cloning or Versatile shuffling). Different expression systems can be used where each cellulosome subunit lays onto a separate vector (single expression system) or the sequences of multiple components are coexpressed and produced by a single host.

Further, different modes of designer cellulosome assembly are possible, such as the "*in vitro*" assembly by co-incubation of different subunits following their individual expression. The assembled complex can then be characterized by native polyacrylamide gel electrophoresis (PAGE) or affinity pull-down. An upgraded version of this process involves the direct assembly on the surface of the expression strain, usually an industrial microorganism, with the ability to directly convert the released sugars to bio-based products, such as ethanol, butanol, etc. (Lamote et al., 2023).

So far mostly yeast and solventogenic clostridia have been engineered to display the cellulosomes, particularly *Saccharomyces cerevisiae* (Tsai et al., 2009; Tsai et al., 2010; Tsai et al., 2013; Fan et al., 2020; Anandharaj et al., 2020, Ma et al., 2024; Sharma et al., 2022), *Pichia pastoris* (Dong et al., 2020) and *Clostridium acetobutylicum* (Kovács et al., 2013; Willson et al., 2016), although engineering of some other industrially relevant microorganisms, such as *Corynebacterium glutamicum* (Lee et al., 2022), has also been reported. Apart from pure solvent-producing cultures, engineered yeast consortium-producing functional mini-cellulosomes have also been reported (Goyal et al., 2021).

Biotechnological applications of designer cellulosomes

The ability to tailor cellulosomes for specific substrates and optimize their efficiency and stability has significant implications for various biotechnological applications based on cellulose waste valorization and the production of bio-based products. One of the most prospective fields that may benefit from the designer cellulosome-based catalysts involves second-generation biofuel production, which relies on converting lignocellulose biomass to solvents or hydrogen. The construction of consolidated bioprocessing systems that simultaneously efficiently degrade various lignocellulose substrates and ferment the released sugars to value-added solvents, like ethanol or butanol, holds significant promise for future sustainable bioprocessing, as it reduces process complexity, minimizes production costs, and enhances overall efficiency (Li et al., 2024). By integrating enzyme production, substrate hydrolysis, and fermentation into a single step, these systems can enable the economically viable conversion of renewable biomass into biofuels and biochemicals, contributing to the development of biorefineries and reducing dependence on fossil fuels (Wen et al., 2020). Furthermore, the trans-

formation of lignocellulose to other value-added products, including different bio-based chemicals (i.e. lactate, succinate), also holds great promise (Liu et al., 2021). In addition, applications in other, more traditional industries, such as textile, paper, feed, and food industries, where (partial) degradation of (hemi)cellulose material enhances the efficiency of processes (e.g., polishing of textiles, deinking of paper sludge, or increasing the availability of high-nutrient molecules), may also be improved through the use of these intricate nanomachines.

Challenges and Future Perspectives

Cellulosomes represent a remarkable natural solution for lignocellulosic biomass degradation, offering a powerful tool for sustainable biotechnology. Their intricate structure, enzymatic synergy, and adaptability to diverse substrates make them ideal for applications ranging from waste management to biofuel production (Xin et al., 2019). However, the construction and industrial application of designer cellulosomes presents several challenges, primarily due to the complexity of designing and assembling these highly specialized enzymatic systems. Key hurdles include the difficulty in optimizing the interactions between the scaffoldin and enzymatic components, ensuring stability and activity under industrial conditions and the need for precise control over the composition and architecture of the complexes. Furthermore, the scalability of designer cellulosome-displaying catalysts for large-scale applications, such as in biofuel production or industrial biomass degradation, remains a significant obstacle (Wen et al., 2020). By improving enzyme efficiency, tailoring cellulosome designs for specific substrates, and optimizing production systems, these biocatalytic nanomachines can significantly contribute to the development of sustainable utilization of lignocellulosic biomass (Ye et al., 2024). While challenges remain, ongoing advances in genetic engineering, synthetic biology, and process optimization promise to unlock their full potential, driving progress in renewable energy and circular bio-economy.

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Conflicts of Interest

The author declares no conflict of interest.

References

Alves, V.D., Fontes, C.M.G.A., Bule, P., 2021. Cellulosomes: Highly efficient cellulolytic complexes. Subcellular Biochemistry, 96, 323–354. https://doi.org/10.1007/978-3-030-58971-4_9

Anandharaj, M., Lin, Y.-J., Rani, R.P., Nadendla, E.K., Ho, M.-C., Huang, C.-C., Cheng, J.-F., Chang, J.-J., Li, W.-H., 2020. Constructing a yeast to express the largest cellulosome complex on the cell surface. Proceedings of the National Academy of Sciences, 117(5), 2385–2394. https://doi.org/10.1073/pnas.1916529117

Arfi, Y., Shamshoum, M., Rogachev, I., Peleg, Y., Bayer, E.A., 2014. Integration of bacterial lytic polysaccharide monooxygenases into designer cellulosomes promotes enhanced cellulose degradation. Proceedings of the National Academy of Sciences of the USA, 111(25), 9109–9114. https://doi.org/10.1073/pnas.1404148111

Artzi, L., Bayer, E.A., Moraïs, S., 2017. Cellulosomes: bacterial nanomachines for dismantling plant polysaccharides. Nature Reviews Microbiology, 15(2), 83–95. https://doi.org/10.1038/nrmicro.2016.164

Asemoloye, M.D., Bello, T.S., Oladoye, P.O., Gbadamosi, M.R., Babarinde, S.O., Adebami, G.E., Olowe, O.M., Temporiti, M.E.E., Wanek, W., Marchisio, M.A., 2023. Engineered yeasts and lignocellulosic biomaterials: shaping a new dimension for biorefinery and global bioeconomy. Bioengineered, 14(1), 2269328. https://doi.org/10.1080/21655979.2023.2269328

Bayer, E.A., Belaich, J.P., Shoham, Y., Lamed, R., 2004. The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. Annual Review of Microbiology, 58, 521–554. https://doi.org/10.1146/annurev.micro.57.030502.091022

Bayer, E.A., Chanzy, H., Lamed, R., Shoham, Y., 1998. Cellulose, cellulases and cellulosomes. Current Opinion in Structural Biology, 8(5), 548–557. https://doi.org/10.1016/S0959-440X(98)80143-7

Caspi, J., Barak, Y., Haimovitz, R., Irwin, D., Lamed, R., Wilson, D.B., Bayer, E.A., 2009. Effect of linker length and dockerin position on conversion of a *Thermobifida fusca* endoglucanase to the cellulosomal mode. Applied and Environmental Microbiology, 75(23), 7335–7342. https://doi.org/10.1128/AEM.01241-09

Chen, C., Cui, Z., Song, X., Liu, Y.J., Cui, Q., Feng, Y., 2016. Integration of bacterial expansin-like proteins into cellulosome promotes cellulose degradation. Applied Microbiology and Biotechnology, 100(5), 2203–2212. https://doi.org/10.1007/s00253-015-7071-6

Dong, C., Qiao, J., Wang, X., et al., 2020. Engineering *Pichia pastoris* with surface-display minicellulosomes for carboxymethyl cellulose hydrolysis and ethanol production. Biotechnology for Biofuels, 13, 108. https://doi.org/10.1186/s13068-020-01749-1

Fan, L.H., Zhang, Z.J., Yu, X.Y., Xue, Y.X., Tan, T.W., 2012. Self-surface assembly of cellulosomes with two miniscaffoldins on *Saccharomyces cerevisiae* for cellulosic ethanol production. Proceedings of the National Academy of Sciences of the USA, 109, 13260–13265. https://doi.org/10.1073/pnas.1209856109

Fierobe, H.P., Mingardon, F., Mechaly, A., Bélaïch, A., Rincon, M.T., Pagès, S., Lamed, R., Tardif, C., Bélaïch, J.P., Bayer, E.A., 2005. Action of designer cellulosomes on homogeneous versus complex substrates: controlled incorporation of three distinct enzymes into a defined trifunctional scaffoldin. Journal of Biological Chemistry, 280(16), 16325–16334. https://doi.org/10.1074/jbc.M414449200

Fierobe, H.P., Mechaly, A., Tardif, C., Belaich, A., Lamed, R., Shoham, Y., Belaich, J.P., Bayer, E.A., 2001. Design and production of active cellulosome chimeras: Selective incorporation of dockerin-containing enzymes into defined functional complexes. Journal of Biological Chemistry, 276(27), 21257–21261. https://doi.org/10.1074/jbc.M102082200

Gayathri, R., Mahboob, S., Govindarajan, M., Al-Ghanim, K.A., Ahmed, Z., Al-Mulhm, N., Vodovnik, M., Vijayalakshmi, S., 2021. A review on biological carbon sequestration: A sustainable solution for a cleaner air environment, less pollution and lower health risks. Journal of King Saud University – Science, 33, 101282. https://doi.org/10.1016/j.jksus.2020.101282

Goyal, G., Tsai, S.L., Madan, B., Dasilva, N.A., Chen, W., 2011. Simultaneous cell growth and ethanol production from cellulose by an engineered yeast consortium displaying a functional mini-cellulosome. Microbial Cell Factories, 10, 89. https://doi.org/10.1186/1475-2859-10-89

Joseph, R.C., Kim, N.M., Sandoval, N.R., 2018. Recent developments of the synthetic biology toolkit for *Clostridium*. Frontiers in Microbiology, 9, 1. https://doi.org/10.3389/fmicb.2018.00741

Khan, A., Moraïs, S., Chung, D., Sarai, N.S., Hengge, N.N., Kahn, A., Himmel, M.E., Bayer, E.A., Bomble, Y.J., 2020. Glycosylation of hyperthermostable designer cellulosome components yields enhanced stability and cellulose hydrolysis. FEBS Journal, 287(20), 4370–4388. https://doi.org/10.1111/febs.15251

Kovács, K., Willson, B.J., Schwarz, K., Heap, J.T., Jackson, A., Bolam, D.N., Winzer, K., Minton, N.P., 2013. Secretion and assembly of functional mini-cellulosomes from synthetic chromosomal operons in Clostridium acetobutylicum ATCC 824. Biotechnology for Biofuels, 6, p.117. https://doi.org/10.1186/1754-6834-6-117

Lamote, B., da Fonseca, M.J.M., Vanderstraeten, J., Meert, K., Elias, M., Briers, Y., 2023. Current challenges in designer cellulosome engineering. Applied Microbiology and Biotechnology, 107(9), 2755–2770. https://doi.org/10.1007/s00253-023-12474-8

Lee, M.E., Ko, Y.J., Hwang, D.H., Cho, B.H., Jeong, W.Y., Bhardwaj, N., Han, S.O., 2022. Surface display of enzyme complex on Corynebacterium glutamicum as a whole-cell biocatalyst and its consolidated bioprocessing using fungal-pretreated lignocellulosic biomass. Bioresource Technology, 362, 127758. https://doi.org/10.1016/j.biortech.2022.127758

Levi Hevroni, B., Moraïs, S., Ben-David, Y., Morag, E., Bayer, E.A., 2020. Minimalistic cellulosome of the butanologenic bacterium Clostridium saccharoperbutylacetonicum. mBio, 11(2), e00443-20. https://doi.org/10.1128/mBio.00443-20

Li, Z., Waghmare, P.R., Dijkhuizen, L., Meng, X., Liu, W., 2024. Research advances on the consolidated bioprocessing of lignocellulosic biomass. Engineering Microbiology, 4(2), 100139. Available at: https://www.sciencedirect.com/science/article/pii/S266737032400002X

Liu, Y., Tang, Y., Gao, H., Zhang, W., Jiang, Y., Xin, F., Jiang, M., 2021. Challenges and future perspectives of promising biotechnologies for lignocellulosic biorefinery. Molecules, 26(17), 5411. https://doi.org/10.3390/molecules26175411

Ma, X.Y., Coleman, B., Prabhu, P., Yang, M., Wen, F., 2024. Engineering compositionally uniform yeast whole-cell biocatalysts with maximized surface enzyme density for cellulosic biofuel production. ACS Synthetic Biology, 13(4), 1225–1236. https://doi.org/10.1021/acssynbio.3c00669

Ponsetto, P., Sasal, E.M., Mazzoli, R., Valetti, F., Gilardi, G., 2024. The potential of native and engineered Clostridia for biomass biorefining. Frontiers in Bioengineering and Biotechnology, 12, 1423935. https://doi.org/10.3389/fbioe.2024.1423935

Sharma, J., Kumar, V., Prasad, R., Gaur, N.A., 2022. Engineering of Saccharomyces cerevisiae as a consolidated bioprocessing host to produce cellulosic ethanol: Recent advancements and current challenges. Biotechnology Advances, 56, 107925. https://doi.org/10.1016/j.biotechadv.2022.107925

Stern, J., Moraïs, S., Lamed, R., Bayer, E.A., 2016. Adaptor scaffoldins: an original strategy for extended designer cellulosomes, inspired from nature. mBio, 7(2), e00083.

Tsai, S., Oh, J., Singh, S., Chen, R., Chen, W., 2009. Functional assembly of minicellulosomes on the Saccharomyces cerevisiae cell surface for cellulose hydrolysis and ethanol production. Applied and Environmental Microbiology, 75, 6087–6093. https://doi.org/10.1128/AEM.01538-09

Tsai, S., Goyal, G., Chen, W., 2010. Surface display of a functional minicellulosome by intracellular complementation using a synthetic yeast consortium and its application to cellulose hydrolysis and ethanol production. Applied and Environmental Microbiology, 76. https://doi.org/10.1128/AEM.01777-10

Tsai, S.-L., DaSilva, N.A., Chen, W., 2013. Functional display of complex cellulosomes on the yeast surface via adaptive assembly. ACS Synthetic Biology, 2, 14–21. https://doi.org/10.1021/sb300047u

Vanderstraeten, J., da Fonseca, M.J.M., De Groote, P., Grimon, D., Gerstmans, H., Kahn, A., Moraïs, S., Bayer, E.A., Briers, Y., 2022a. Combinatorial assembly and optimisation of designer cellulosomes: a galactomannan case study. Biotechnology for Biofuels and Bioproducts, 15(1), 60. https://doi.org/10.1186/s13068-022-02158-2

Vazana, Y., Barak, Y., Unger, T., Peleg, Y., Shamshoum, M., Ben-Yehezkel, T., Mazor, Y., Shapiro, E., Lamed, R., Bayer, E.A., 2013. A synthetic biology approach for evaluating the functional contribution of designer cellulosome components to deconstruction of cellulosic substrates. Biotechnology for Biofuels, 6(1), 182. https://doi.org/10.1186/1754-6834-6-182

Vazana, Y., Moraïs, S., Barak, Y., Lamed, R., Bayer, E.A., 2010. Interplay between *Clostridium thermocellum* Family 48 and Family 9 cellulases in cellulosomal versus noncellulosomal states. Applied and Environmental Microbiology, 76. https://doi.org/10.1128/AEM.00009-10

Vodovnik, M., Duncan, S.H., Reid, M.D., Cantlay, L., Turner, K., Parkhill, J., et al., 2013. Correction: Expression of cellulosome components and type IV pili within the extracellular proteome of *Ruminococcus flavefaciens* 007. PLoS ONE, 8(12), 10.1371/annotation/fed83700-d3cd-428e-ae52-e60524c97529. https://doi.org/10.1371/annotation/fed83700-d3cd-428e-ae52-e60524c97529

Wen, Z., Li, Q., Liu, J., Jin, M., Yang, S., 2020. Consolidated bioprocessing for butanol production of cellulolytic Clostridia: development and optimization. Microbial Biotechnology, 13(2), 410–422. https://doi.org/10.1111/1751-7915.13478

Wen, F., Sun, J., Zhao, H., 2010. Yeast surface display of trifunctional minicellulosomes for simultaneous saccharification and fermentation of cellulose to ethanol. Applied and Environmental Microbiology, 76. https://doi.org/10.1128/AEM.01687-09

Willson, B.J., Kovács, K., Wilding-Steele, T., Markus, R., Winzer, K., Minton, N.P., 2016. Production of a functional cell wall-anchored minicellulosome by recombinant *Clostridium acetobutylicum* ATCC 824. Biotechnology for Biofuels, 9, 109. https://doi.org/10.1186/s13068-016-0523-7

Xin, F., Dong, W., Zhang, W., Ma, J., Jiang, M., 2019. Biobutanol production from crystalline cellulose through consolidated bioprocessing. Trends in Biotechnology, 37, 167–180. https://doi.org/10.1016/j.tibtech.2018.09.003

Ye, Y., Liu, H., Wang, Z., Qi, Q., Du, J., Tian, S., 2024. A cellulosomal yeast reaction system of lignin-degrading enzymes for cellulosic ethanol fermentation. Biotechnology Letters, 46(4), 531–543. https://doi.org/10.1007/s10529-024-03485-0

5. slovensko posvetovanje mikroskopistov

Slovensko društvo za mikroskopijo (https://sdm.mikroskopsko-drustvo.si/) že od leta 2015 redno organizira znanstveno-strokovna srečanja z naslovom 'Slovensko posvetovanje mikroskopistov'. Tako je 16. in 17. maja 2024 na Rogli potekalo že 5. slovensko posvetovanje mikroskopistov, povzetki vseh prispevkov so dostopni na strani: https://www.mikroskopsko-drustvo.si/posvet/knjiga-povzetkov-book-of-abstracts/. Srečanje je sooblikovalo 121 udeležencev - raziskovalci, študenti in drugi strokovnjaki, ki delujejo na različnih področjih znanosti o življenju in znanosti o materialih, ter razstavljavci opreme in aparatur s področja mikroskopije. Predsednica Slovenskega društva za mikroskopijo, dr. Kristina Žagar Soderžnik, je v pozdravnem nagovoru izpostavila pomen srečanja za prenos znanja o sodobnih metodologijah na področju mikroskopije, za povezovanje raziskovalcev iz različnih znanstvenih področij ter za izmenjavo izkušenj z uporabniki mikroskopskih analiz v raziskovalno-razvojnih, diagnostičnih, industrijskih ter drugih laboratorijih in ustanovah.

Nove metodologije, izzive in rezultate svojega dela so predstavili raziskovalci iz različnih inštitutov, univerz in podjetij iz Slovenije ter nekaj gostov iz tujine, ki intenzivno sodelujejo z našimi laboratoriji. V okviru dveh plenarnih predavanj smo razpravljali o meritvah energijske presnove astrocitov z gensko kodiranimi senzorji na osnovi prenosa energije z resonanco fluorescence (dr. Marko Kreft, Univerza v Ljubljani BF in MF, Celica d.o.o.) in o raziskavah perovskitnih feroelektrikov z *in situ* presevno elektronsko mikroskopijo (dr. Andreja Benčan Golob, Inštitut Jožef Stefan, Ljubljana). V sedmih vabljenih predavanjih smo se seznanili z razvojem in uporabo mikroskopskih analiz v kombinaciji z drugimi metodami na raznolikih področjih: v študijah komunikacije med celicami na osnovi tunelskih membranskih nanocevk, v analizah vpliva podnebnih sprememb na rast dreves, v razvoju novih pristopov za zdravljenje raka z uporabo elektroporacije, v razvoju raznolikih uporab 3D rentgenskega slikanja, v razvoju in kontroli kakovosti v industriji vrhunskih izdelkov iz aluminija, v raziskavah elektrokemijskih reakcij z *in situ* presevno elektronsko mikroskopijo in v naprednih postopkih za karakterizacijo materialov s katodoluminiscenco. V dveh dneh se je zvrstilo še 56 predstavitev s posterji in 14 krajših predavanj o novostih na področju priprave in mikroskopskih analiz vzorcev, kar je spodbudilo zelo živahne razprave o novih metodoloških možnostih, ki so na razpolago v laboratorijih v Sloveniji, o najnovejšem razvoju metodologije v globalnih raziskavah in o spektru možnih uporab novih tehnik. V znanostih o življenju je zelo aktualno področje razvoja različnih pristopov za integracijo in korelacijo več načinov slikanja, ki so komplementarni z vidika različnih vrst pridobljenih informacij, različnih ločljivosti in velikosti analiziranega vzorca. Inovativni postopki integrativne mikroskopije bioloških objektov nam omogočajo celovit vpogled v biološke strukture in procese. Poleg tega je za pridobivanje kvantitativnih rezultatov iz slikovnih podatkov ključnega pomena napredna analiza slik. Srečanje smo zaključili z razpravo o pomembnem dogodku, ki ga Slovensko društvo za mikroskopijo organizira v naslednjem letu, to je mednarodni kongres 17th Multinational Congress on Microscopy (https://17mcm.si/).

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