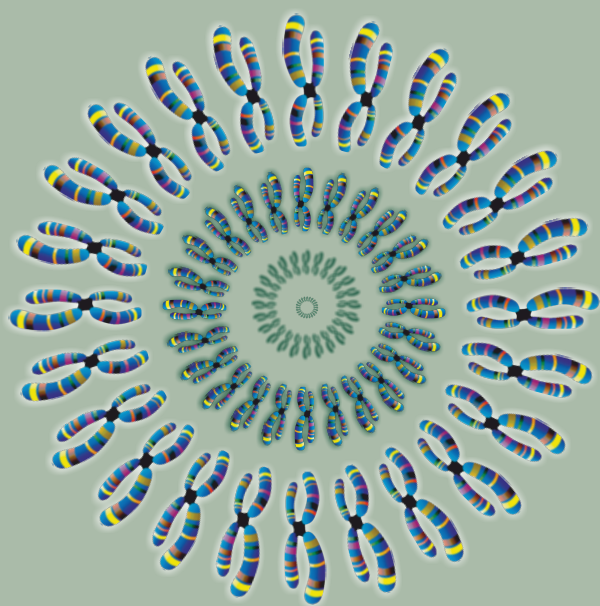
ALEXANDRU IOAN CUZA UNIVERSITY OF IASI

JOURNAL OF EXPERIMENTAL AND MOLECULAR BIOLOGY

Volume 25 Number 3 (2024)

ISSN 2601 – 6974



Alexandru Ioan Cuza University Publishing House

„ALEXANDRU IOAN CUZA” UNIVERSITY OF IAȘI

JOURNAL OF EXPERIMENTAL AND MOLECULAR BIOLOGY

TOME XXV, Number 3 **2024**

Editura Universității „ALEXANDRU IOAN CUZA” din Iași

EDITORIAL OFFICE

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Laboratorul de Biochimie și Biologie Moleculară
Bulevardul Carol I, Nr. 20A, 700506, Iași, România
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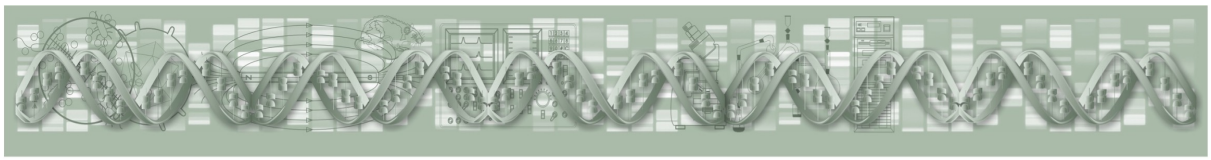

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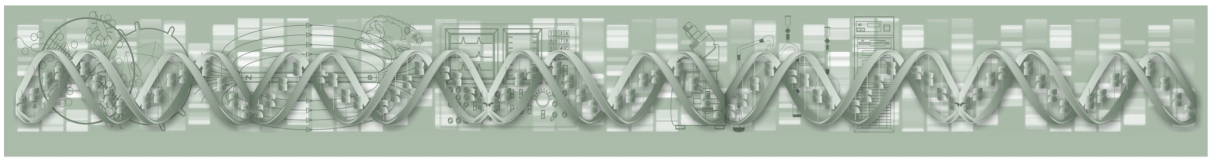

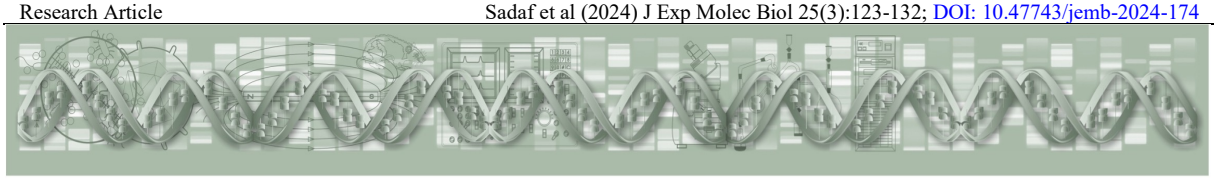

APPLICATIONS OF PHYTOHORMONES FOR *CANNABIS SATIVA* CALLUS, SHOOT, AND ROOT INDUCTION UNDER TISSUE CULTURE TECHNIQUE

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Abstract

Cannabis sativa in vitro cultivation interest has been invigorated/renewed due to health and economic benefits, unconventional strategy, quality plants, and industrial fiber. The in vitro regeneration of cannabis encountered establishment and disinfection aspects globally. The research on plant growth regulator composition, combination, concentrations, decontamination, significant influence on, callus, shoot, and root induction, and regeneration is needed to shed light on desirable, healthy plants for the cannabis industry. The study outcomes indicated some disinfectant duration and concentrations exposure to ex-plant have a toxic impact and reduced growth while 5 % sodium hypochlorite and mercury chloride 0.1-1% are efficient sterile and gave good seed germination. Sufficient increase in callus proliferation was noticed on combination and concentrations of MS (Murashige and Skoog) media treated with 450 µl kinetin +450 ml BAP+450 µl 2,4-D from cannabis sativa leaves. Substantial influence on shoot survival was recorded on MS media supplemented with increasing 2-3 ml BAP concentration. Maximum rhizogenesis observed on PGR (plant growth regulator) 5ml (indole-3-acetic acid) IAA and 8ml (indole-3-butyric acid) IBA combinations. The study output suggests that in vitro cultivation using phytohormones protocols is applicable and appropriate for cannabis survival and productivity and can be valuable for medical and industrial purposes.

Keywords: *Cannabis sativa*, Murashige and Skoog, indole-3-acetic acid, Kinetin 2,4D, BAP, Kinetin, IAA, IBA

Introduction

Cannabis sativa, an annual plant with herbal flora, belongs to the family of Cannabaceae. The common name is hemp, natively to Eastern Asia but currently cultivated worldwide. In 1753, Carl Linnaeus first classified the sativa and believed to be as a cross-pollinator plant. Various environmental factors contribute to cannabinoid growth and development [1]. Cannabis is an adaptable herb with noticeable pharmacologic characteristics, which produces several medicines depending on the composition of cannabinoids and phytochemicals. Tetrahydrocannabinol (THC) is a well-known plant cannabinoid that is an essential agent for antinausea and muscle relaxation. Currently, cannabidiol (CBD) is an available product in the market. The ratios of tetrahydrocannabinol/Cannabidiol (THC/CBD) were genetically established. The female plants produce enough THC for drug production. *Cannabis sativa* has a vital role in the field of medicine, such as enhancing lung capacity, relief of chronic pain, fighting cancer, regulating and preventing diabetes, helping to cure depression, and alleviating anxiety. Clinically, cannabis is involved in the prevention of cancer. Cannabis is used for the

Received 6 February 2024; Revised 24 May 2024; Accepted 25 June 2024;
Published Online 26 June 2024; Published 9 October 2024

medication of nausea/diarrhea, to improve appetite in AIDS/HIV patients, and to relieve severe body pain and spasms. District of Columbia and thirty-three states legally allow cannabis for their utilization. In 1996, California first legalized cannabis for medical use [2]; [3]. In China, *Cannabis sativa* has been cultivated as a fiber, food, and medicine. China is the oldest country for cannabis cultivation, bringing different germplasm with hereditarily unmistakable local assortments of fiber-rich cannabis. Cultivation of plant cells and tissues mediates aseptic controlled nutrient culture media to expand plant cells, tissues, and organs [4]. *In vitro* culture methods are vital for creating infection-free plants, speedy growth for uncommon plant genotypes, plant genome alteration, and creation of plant-inferred metabolites of significant business value. There is a minimal amount of tissue culture studies on *Cannabis sativa*. According to the increased demand for cannabis in the field of medicine, the rate of cannabis through micropropagation is the most efficient and reliable. Different factors such as medium, stock solution concentration, light, pH, temperature, concentration of sucrose, and type of hormones can affect seed growth under in vitro conditions (Shi et al. 2024). The concentration of phytohormones influences the induction of shoots, callus, meristem, root, and multiplication during *in vitro* regeneration of plants (Page et al. 2020; Sharma et al. 2013). Some of the mainly utilized phytohormones include (BAP) 6-benzylaminopurine, cytokinins, (TDZ) thidiazuron, (ZT) zeatin, (KN) kinetin, auxins, and (mT) meta-Topolin, (NAA) naphthalene acetic acid, (IBA) indole butyric acid, indole acetic acid (IAA) and (2,4-D) 2,4-dichlorophenoxy acetic acid.

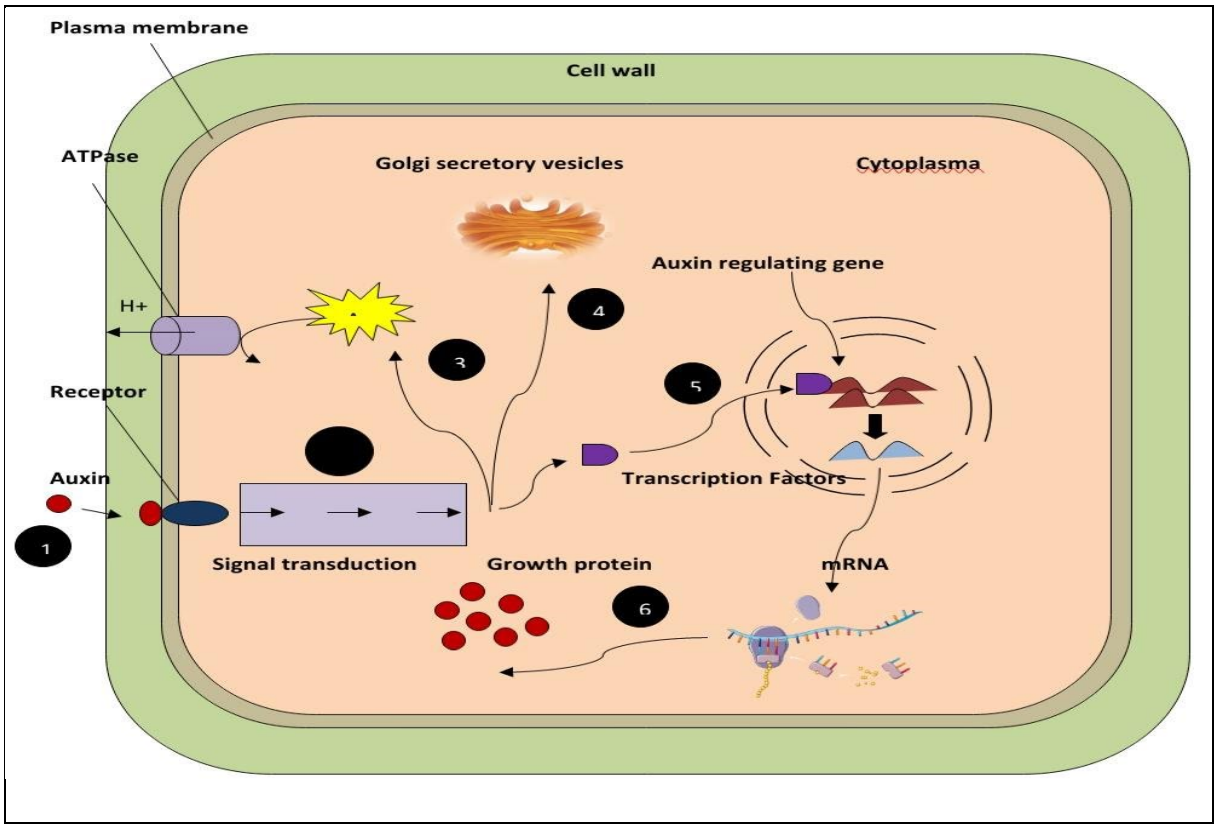


Figure 1. Signalling pathway of auxin in the plant cell (Bhatla and Lal. 2023)

The study of Miller et al. (1955), Truta et al. (2011) proposed that kinetin from autoclaved herring sperm, where cell division is stimulated by plant growth regulator (2,4-D) dichlorophenoxyacetic acid, and (auxin) in low-concentration and an efficient regulator of large-leaf plant development. In (Miller et al. 1955) study, different phytohormones such as (BAP) 6-benzylaminopurine, Kinetin, (2,4D) (2,4-Dichlorophenoxy acetic acid, (IAA) Indole-3-acetic acid were used for the plant induction and proliferation, for the induction of callus

(myoinositol, sugar, stock solution, BAP, kinetin, 2, 4 D, and celery gel) is used as a media. For callus media, the concentration of BAP is used in microliters. BAP is essential for plant growth and development responses, cell division, shoot elongation, flowering, and plant fruiting. For root and shoot induction, 3ml BAP was added in 1L for fast growth [5].

Materials and methods

Sample collection

In the current study, the seeds of disease-free cannabis were taken from two cities in Pakistan, D.G Khan and Lahore, and the collected seeds were placed within controlled conditions in the culture room of the tissue culture lab.

Washing and sterilization of seeds

Seeds were washed in a series of disinfectants to eliminate all dust particles. The seeds were dipped in tap water for 30 minutes, 70% ethanol manufactured by Unicol limited for 2 minutes. The ethanol residues were removed and washed with deionized distilled autoclaved water. After washing detergent for 4 to 5 minutes used and seeds were rewashed with autoclaved double-distilled water to remove detergent residues. Seeds were further treated with Unichem chemicals supplier's mercury chloride and 5% sodium hypochlorite manufactured by Ittehad Chemicals Limited. Finally, three times seeds were washed with autoclave deionized distilled water and sterilized seeds were ready for further experiment.

Seed inoculation media and conditions

Sterilized seeds of both varieties (Lahore and DG Khan) were inoculated on (Murashige and Skoog 1962) MS media petri plates under a BIOBASE laminar flow cabinet. Polypropylene sheets tightly covered petri plates and placed in the tissue culture room (temperature 22-28° C, humidity 30-40%, light 5000 lux).

Callus induction preparations

For callus induction three weeks old 0.6 cm cannabis leaves grown in tissue culture lab, were cut into small pieces with the help of a scalpel blade, and sterilized with (Ioannidis et al. 2022) method under laminar flow cabinet. The sterile leaves were inoculated in MS media incorpated PGR, BAP manufactured by EMCO Industries Ltd, Bayer 2,4 D, and AdooQ® Bioscience kinetin with various combination and concentrations include 50µl BAP,kinetin 25µl, 2, 4D 25µl, combine treatement of 50µl BAP + 25µl 2, 4D, 50µl kinetin + 50µl BAP +50µl 2, 4D, BAP 250µl +250µl kinetin, 50µl kinetin + 50µl BAP, 450µl + 450µl 2, 4D and kinetin 450µl+ BAP 450ml+ 2, 4D 450µl was added in series of flask and pH was set on 5.7 by using ChromTech pH meter. After addation of growth regulators combinations put solidifying agent 3.5g celery gel made of EMCO Industries Ltd. The sterile leaf pieces were inoculated in media under a laminar flow cabinet with the help of sterilized forceps, and flasks were covered tightly with polypropylene sheets and rubber bands and placed in a tissue culture room (temperature 22-28 ° C, humidity 30-40%, light 5000 lux). after 20 days of culturing callus efficiency (%), length (cm), growth rate, and color indication were evaluated.

Shoot induction media and conditions

Leaves derived from callus were cut into small pieces by a scalpel blade under sterile conditions. adjusted 5.7 pH of media and autoclaved in BIOBASE manufacture autoclave for 1 hour at 121°C. Callus small pieces were inoculated on cotton dipped in semi-solid media with three different BAP (2ml BAP, 2.5ml BAP, and 3ml BAP) concentrations. Flasks were put in a tissue culture lab under a controlled environment (temperature 22-28 ° C, humidity 30-40%, light 5000 lux). the results were described by evaluating shoot length, shoot efficiency (%), color, and growth rate.

Root induction

Shoots of cannabis 0.5 cm were cut into pieces with the help of a scalpel blade in laminar flow under sterile conditions for the induction of root. Shoots were inoculated on MS media treated with combination and concentrations of root growth regulators 5ml IAA, 8ml IBA, and combination IAA + IBA. After inoculation, flasks were placed under a controlled environment (temperature 22-28 ° C, humidity 30-40%, light 5000 lux) in a tissue culture room. Root efficiency (%), length (cm), and growth rate were evaluated.

Statistical analysis

The phytohormone concentration and combination were applied in three replicates and analyzed by using Microsoft Excel software and Tukey's significant differences for variance ANOVA and LSD to compare mean values.

Results

Seed germination of *Cannabis sativa* from two different areas

Both seed varieties were sterilized with 0.1-1% mercury chloride and 5% sodium hypochlorite for 5 minutes and inoculated on MS media. The Lahore seeds did not show germination due to immaturity after one week and D.G Khan's seed growth in the medium was three leaves and 4cm tall.

Figure 2. The image (a,b,d) showed cannabis seed sterilization and (c,e,f,g) germination of seeds under in vitro conditions

Sterilization chemicals used in cannabis tissue culture preparation. Disinfectants for cannabis tissue culture germination To culture the apical meristem and seeds of cannabis it's sterilized with different detergents to remove all the possible contaminants sterilized 2-5 times with tap water, and distilled water, 10-20 minutes with one drop of tween 20 into 100 ml of water and washing detergent 2-4 drops was used for 5-15 minutes in the second step but growth did not occur. After the failure of attempts, 5% sodium hypochlorite for 5-30 minutes and germination was observed. Mercury chloride 0.1-1% for 2-10 minutes and good seed germination was noticed as compared to sodium hypochlorite treatment.

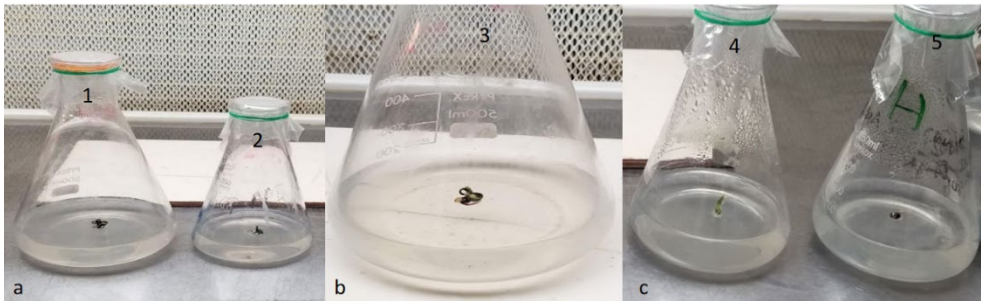


Figure 3. Represented the impact of sterilizing agent in cannabis tissue culture. In image (a,b) flask 1,3 was treated with washing detergent, flask 2 with tween twenty, in (c) image flask 4,5 supplemented with sodium hypochlorite and mercury chloride

Growth regulators combination and concentrations for callus induction

In Table 1, various combinations and concentrations of growth regulators were supplemented to the callus media to check the callus growth rate and suitable concentrations and combinations of callus. 50µl BAP, kinetin 25µl and 2, 4D 25µl treatments showed no callus growth. The combination of 50µl BAP + 25µl 2, 4D represented asmall amount of blackish colour callus growth after 20 days with low growth rate, combine treatment of 50µl kinetin + 50µl BAP +50µl 2, 4D indicated a greenish colour callus start grow within 20 days with good growth rate. BAP 250 µl +250 µl kinetinshowed low callus growth rate , the 50 µl kinetin + 50 µl BAP represented low growth rate of callus, kinetin 450 µl + 450 µl 2, 4D showed a very small amount of dark red colour callus growth within 20 days and kinetin 450 µl+ BAP 450 ml+ 2, 4D 450 µl showed very good amount of dark green colour callus growth on the callus media within 20 days.

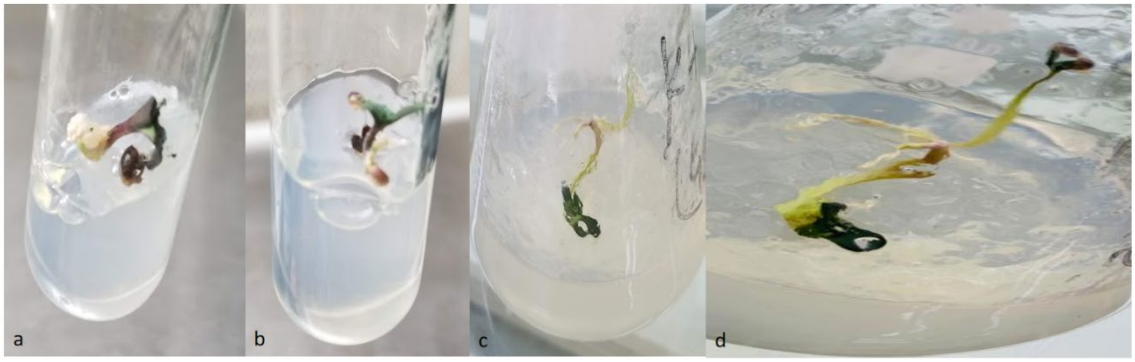


Figure 4. The image (a,b,c,d) indicated callus growth with concentration of hormones combinations

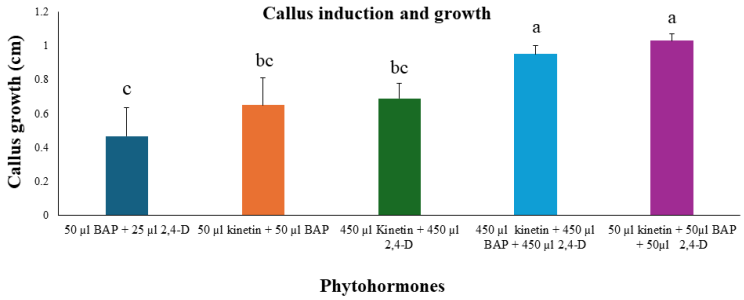


Figure 5. *Cannabis sativa* callus induction and growth by different phytohormone concentrations. The figure showed various lowercase letters with significant differences ($P<0.05$) among treatments and means ± standard error

Impact of BAP for shoot induction BAP combination and concentration alone and combined behave differently on cannabis shoots for 25 days. The results indicated increasing the concentration of BAP improves shoot efficiency. At 2 ml BAP shoots were grown with a small growth rate, 2.5ml BAP showed shoots were grown with good growth rate, and 3 ml BAP concentration represented highest shoots growth on shoot media.

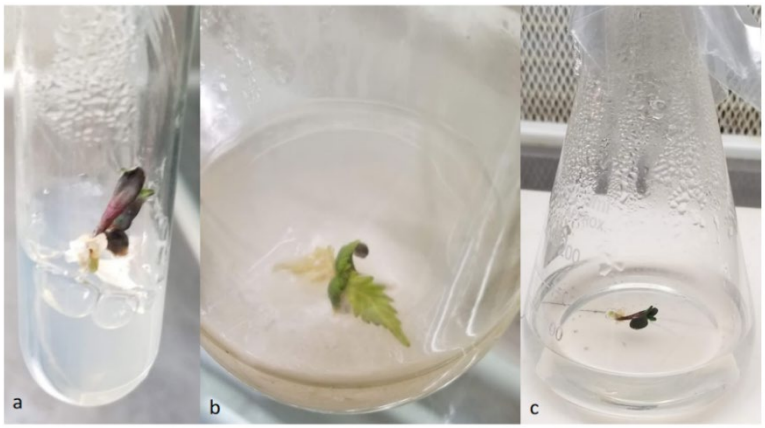


Figure 6. Effect of various BAP concentrations on Cannabis sativa shoot growth. Image (a) represented shoot regeneration supplemented with shoot media 2.5ml BAP, (b) 3ml BAP, and (c) 2ml BAP

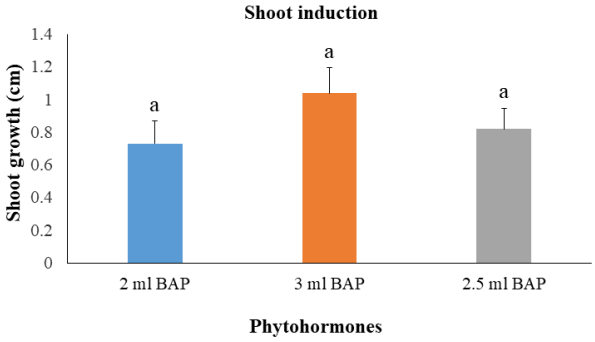


Figure 7. Shoot induction and regeneration with different BAP concentrations and lowercase letters showed significant differences ($P<0.05$) among treatments and means ± standard error

Growth regulators combination for root induction

Roots were inoculated on MS media incorporated root growth regulators 5ml IAA resulted in minimal root induction, 8ml IBA addition illustrated moderated root induction after 25 days. combinations of both IAA+IBA were added for root induction and maximum root efficiency was calculated after 25 days.

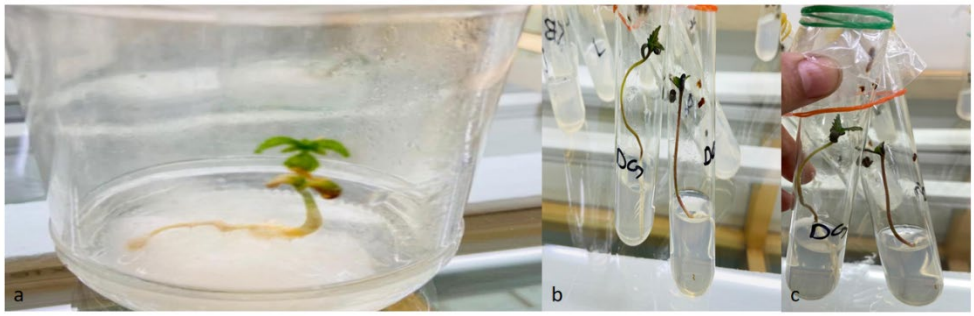


Figure 8. The image (a) illustrates cannabis roots regeneration with 5ml IAA and (b,c) 5ml IAA+ 8ml IBA treatment impacts

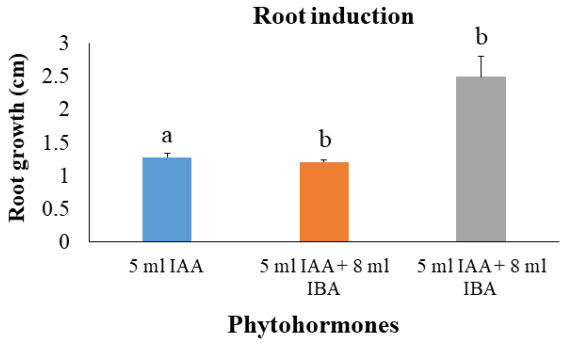


Figure 9. Root induction and growth with different IAA and IBA concentrations and lowercase letters showed significant differences (P<0.05) among treatments and means ± standard error

Table 1. Growth regulators combination and concentrations for callus induction indicated various combinations and concentrations of growth regulators were supplemented to the MS media to check callus, shoot and root induction, growth rate, suitable concentrations, and combinations. The (+ and – are the indications of callus growth (- shows no growth of callus, + shows growth in a tiny amount, ++ good growth, and +++ indicates growth was excellent)

Callus growth regulators	Concentrations	Growth rate	Days
BAP	50 µl	-	20
Kinetin	25 µl	-	20
2,4-D	25 µl	-	20
BAP + 2,4-D	50 µl + 25 µl	+	20
Kinetin + BAP	50 µl + 50 µl	+	20
Kinetin+2,4-D	450 µl + 450 µl	+	20
kinetin + BAP +2,4-D	450 µl + 450 µl+ 450 µl	++	20
kinetin + BAP +2,4-D	50 µl + 50 µl+ 50 µl	+++	20
Shoot growth regulators			
BAP	2 ml	+	25
BAP	2.5 ml	++	25
BAP	3 ml	+++	25
Root growth regulators			
IAA	5 ml	+	25
IBA	8 ml	+	25
IAA + IBA	5 ml+8 ml	+++	25

Discussions

Recent *Cannabis sativa* plants in vitro production due to their medicinal purposes, and economical benefits, to save time and labour force a biotechnological tool success is needed that encountered establishment and disinfection aspects under in vitro regeneration. In this respect, our findings indicated the plant growth regulator media composition, combination, and concentrations, origin of explant, decontamination, had a significant influence on, callus, shoot, and root induction and regeneration which is in agreement with (Feeney and Punja 2003, Wielgus et al. 2008, Lata et al. 2010, Thacker et al. 2018, Monthony et al. 2021).
presented a resemblance with (Manreet et al. 2000, Mishra et al. 2006). The study outcomes illustrated before tissue culture initiation the contamination recognition and address is a crucial

factor that devastated the culture after dissemination and showed a resemblance with (Polivanova and Bedarev 2022). The disinfectant duration and concentrations are utmost factors for ex-plant minimal toxicity and survival. In our study, some sterilizer exposure to ex-plant had a toxic impact and reduced growth while sodium hypochlorite and mercury chloride 0.1-1% exposed plants showed good germination rate and also exhibited similarity with (Burbulis et al. 2015, Sorokin et al. 2022). The present study result callus induction on MS media supplemented with 2,4-D in combination with kinetin proved optimal growth regulator mixtures treatment and produced the greatest callus showed similarity with the findings of (Mandolino and Ranalli 1999, Slusarkiewicz-Jarzina et al. 2005). The 2,4-D several-level treatments enhance callus proliferation supported by (Page et al. 2020) study. Callus-regenerated plants derived from stem ex-plants showed a sufficient increase in MS media treated with various BAP, kinetin, and 2,4-D combinations and concentrations. Such outcomes are by (Yasemin and Beruto 2024).

Our findings indicated that the greater rise observed in shoot growth with increasing concentration of BAP in MS media and comparable with findings of the (Rusea et al. 2018).

The present study discovered a combination of IAA and IBA growth regulators efficiently induced the roots from the shoot of Cannabis. (Yu et al. 2023, Jdaidi et al. 2024) documented that medicinal plants in vitro rooting was significantly enhanced on MS media treated with IBA. Others (Marimuthu and Muthuchelian 2024) are also in favor of the combination of IBA and IAA-mediated root formation.

Conclusions

Cannabis sativa in vitro cultivation by following various disinfection and phytohormones combination and concentration protocols conclude that some sterilizer exposure to ex-plant has a toxic impact and reduced growth while sodium hypochlorite and mercury chloride duration and concentrations exposure have efficient impact on seed germination. In addition, a combination and concentrations of MS media treated with 450 µl kinetin +450 ml BAP+450 µl 2,4-D from cannabis sativa leaves noticed a sufficient increase in callus proliferation. Maximum shoot induction was achieved on MS media containing increasing concentrations of 2-3 ml BAP. The MS media supplemented PGR 5ml IAA and 8ml IBA combination were considered effective for the highest rhizogenesis. As phytohormones combination serves as a promising base for in vitro better cultivation more possibilities are required in protocols formulation of hemp propagation. This fact is interesting for further studies in the regeneration of cannabis plants from callus and metabolic engineering studies. Ongoing working endeavors are crucial in the efficacy enhancement of micropropagation, clean plant productivity, and germplasm preservation.

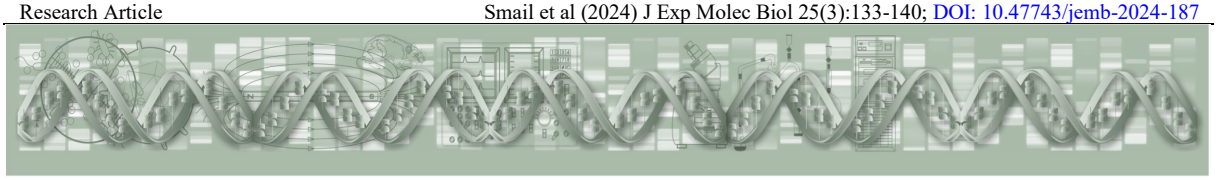
Declarations

Consent for publication: Not applicable
Availability of data and materials: on request by corresponding author
Competing interests: the authors have no competing interest
Funding: no funding for this research
Authors' contributions: all authors contribute equally
Acknowledgements: I am grateful to all of those with whom I have had the pleasure to work during this and other related projects.
Ethical statement: Ethical approval not required for this study

References

Bhatla S.C., Lal M.A. 2023. Auxins. In: *Plant physiology, development and metabolism*. Singapore: Springer Nature Singapore. pp. 399-420.
Burbulis N., Blinstrubienė A., Jonytienė V. 2015. In vitro regeneration from leaf explants of *Petunia hybrida* L. Propagation of Ornamental plants. 15(2): 47-52.
Çördük N. et al. A comparative study of the antimicrobial activities of field-grown and in vitro grown plants of Verbascum scamandri Murb. Advanced and Contemporary Studies in Natural Science and Mathematics: p. 82.
Feeney M., Punja Z.K. 2003. Tissue culture and Agrobacterium-mediated transformation of hemp (*Cannabis sativa* L.). In Vitro Cellular & Developmental Biology-Plant. 39: 578-585.
Gagné V., Boucher N., Desgagné-Penix I. 2024. Cannabis roots: therapeutic, biotechnological and environmental aspects. Cannabis and cannabinoid research.
Jdaidi N., Selmi H., Aloui F., Chaabane A. 2024. In Vitro Conservation of *Prunus Avium*, an Endangered Species in Tunisia, Using a Plant Extract of *Rubus Fruticosus* Compared to Indole-3-Butyric Acid. Plant Protection. 8(01): 33-39.
Jin J., Chen Y., Cai J., Lv L., Zeng X., Li J., ..., Li Y. 2024. Establishment of an efficient regeneration system of 'ZiKui'tea with hypocotyl as explants.
Hesami M., Pepe M., Monthony A.S., Baiton A., Jones A.M.P. 2021. Modeling and optimizing in vitro seed germination of industrial hemp (*Cannabis sativa* L.). Industrial Crops and Products. 170: 113753.
Lata H., Chandra S., Khan I.A., El Sohly M.A. 2010. High frequency plant regeneration from leaf derived callus of high Δ9-tetrahydrocannabinol yielding *Cannabis sativa* L. Planta medica. 76(14): 1629-1633.
Mandolino G., Ranalli P. 1999. Advances in biotechnological approaches for hemp breeding and industry. Advances in hemp research. 9: 185-212.
Manreet S., Arora J.S., Kushal S., Gosal S.S. 2000. Effect of gamma ray irradiation on in vitro multiple shoot formation and establishment of carnation plants. Journal of Ornamental Horticulture (New Series). 3(2): 118-119.
Marimuthu E., Muthuchelian K. 2024. Optimizing callus formation in the endangered medicinal tree *Hydnocarpus macrocarpa* using phytohormone. South African Journal of Botany.168: 340-351.
Miller C.O., Skoog F., Von Saltza M.H., Strong F.M. 1955. Kinetin, a cell division factor from deoxyribonucleic acid1. Journal of the American Chemical Society.77(5): 1392-1392.
Mishra A., Pandey R.K., Sharma J.P., Kumar J. 2006. In vitro propagation of petunia (*Petunia hybrida*) var Cascade Burgundy through multiple shoot culture.
Monthony A.S., Kyne S.T., Grainger C.M., Jones A.M.P. 2021. Recalcitrance of *Cannabis sativa* to de novo regeneration; a multi-genotype replication study. PLoS One.16(8): e0235525.
Monthony A.S., Jones A.M.P. 2024. Enhancing Protoplast Isolation and Early Cell Division from *Cannabis sativa* Callus Cultures via Phenylpropanoid Inhibition. Plants. 13(1): 130.
Murashige T., Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia plantarum. 15(3).
Page S.R.G., Monthony A.S., Jones A.M.P. 2020. Basal media optimization for the micropropagation and callogenesis of *Cannabis sativa* L. BioRxiv. 1: 1-23.
Polivanova O.B., Bedarev V.A. 2022. Hyperhydricity in plant tissue culture. Plants. 11(23): 3313.
Roberts M. 1988. Medicinal products through plant biotechnology. Manipulating Secondary Metabolism in Culture. p. 201-216.

Sharma N.K., Kumar M., Choudhary R. 2013. Effect of 2, 4-D, BAP, KN, IAA and IBA on in vitro Regeneration of Ocimum canum Sims-An Important Hoary Basil Plant. International Journal of Agriculture, Environment and Biotechnology. 6(3): 389-396.
Shi X., Collado C.E., Hernández R. 2024. Improve *Cannabis sativa* micropropagation through increasing air change rate in photoautotrophic and traditional tissue culture. Scientia Horticulturae. 333: 113238.
Slusarkiewicz-Jarzina A., Ponitka A., Kaczmarek Z. 2005. Influence of cultivar, explant source and plant growth regulator on callus induction and plant regeneration of *Cannabis sativa* L. Acta Biol. Crac. Ser. Bot. 47: 145-151.
Sorokin A., Yadav N.S., Gaudet D., Kovalchuk I. 2022. Development and optimization of efficient regeneration and transformation method for drug-type cultivars of *Cannabis sativa*. Department of Biological Sciences, University of Lethbridge, Lethbridge T1K 3M4, Alberta, Canada: 57.
Thacker X., Thomas K., Fuller M., Smith S., DuBois J. 2018. Determination of optimal hormone and mineral salts levels in tissue culture media for callus induction and growth of industrial hemp (*Cannabis sativa* L.). Agricultural Sciences. 9(10): 1250-1268.
Yasemin S., Beruto M. 2024. A Review on Flower Bulb Micropropagation: Challenges and Opportunities. Horticulturae.10(3): 284.
Yu L., Duan Y., Zhan H. 2023. An improved micropropagation of a medicinal plant *Stephania dentifolia*. Plant Cell, Tissue and Organ Culture (PCTOC). 153(1): 219-224.
Wang T. et al. 2008. Adverse effects of medical cannabinoids: a systematic review. Cmaj. 178(13): 1669-1678.
Wielgus K., Luwanska A., LassocinskiW., Kaczmarek Z. 2008. Estimation of *Cannabis sativa* L. tissue culture conditions essential for callus induction and plant regeneration. Journal of Natural Fibers. 5(3): 199-207.



EFFECT OF SEWAGE WASTEWATER ON THE MITOTIC INDEX OF THE ROOT TIPS OF *ALLIUM CEPA* IN KOYA CITY /KURDISTAN REGION OF IRAQ

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Abstract

The purpose of the experiment was to investigate onion (*Allium cepa*) root growth, cell division, and mitotic index. The onions were placed into four different water samples (Control water, Azadi bridge, Sar Razan, and Sar Baskan) for 72 hours in beakers. The results showed that the number of divided cells decreased significantly in the sewage water compared with the control water. The control water had a high percentage of divided cells (18%), whereas the Sar Razan sewage water had the least percentage of divided cells (1.3%). The percentage of divided cells in Sar Baskan sewage water was 2%, and the percentage of divided cells in Azadi Bridge was 2.6%. It was found that the mitotic index and the number of divided cells increased in the control water compared to the sewage water. The results showed that sewage water has a greater impact on root growth, cell division, and mitotic index of root tips of *A. cepa.*
Keywords: *Allium cepa*, sewage water, Root growth, divided cells, mitotic index

Introduction

In most developing nations, the risk of drinking contaminated water and dealing with sanitation issues is growing daily as a result of urbanization and industrialization (Rajasulochana and Preethy 2016). When untreated sewage water is utilized for irrigation in agricultural areas, the contaminated water—which contains hazardous chemical compounds—is immediately dumped into the fields. The contaminated water contains heavy metals, sewage, and other urban garbage. Using water contaminated with microorganisms poses serious health risks, particularly when individuals consume raw vegetables that have been irrigated with the contaminated water (Darlington 1942). Wastes carried by water, in suspension or solution, that drain from a community are referred to as sewage. It is often referred to as the community's used water supply or wastewater flows. Its volume or flow rate, physical state, chemical composition, and the bacteriological organisms it includes all indicate that it is more than 99.9% pure water (Igwenyi 2012).

The onion, or *Allium cepa* L., is a member of the Alliaceae family and genus Allium (Hanelt 2018). The Amaryllidaceae family contains 887 species of *Allium* that are distributed worldwide, encompassing both cultivated and wild plants. *Allium* species are highly significant herbaceous plants that are utilized as vegetables and seasonings around the world. Early taxonomy of angiosperms included Allium and related genera under the Liliaceae family. The monocotyledons are acknowledged as belonging to the unique family Alliaceae, which is closely related to the Amaryllidaceae, in the more modern and competent taxonomic approach (Dahlgren et al. 1985). Since ancient times, it has been prized as a culinary and medicinal plant. It is a vegetable bulb crop that is known to most cultures and consumed all over the world,

Received 26 March 2024; Reviewed 23 April 2024; Accepted 26 June 2024;
Published Online 27 June 2024; Published 9 October 2024

produced to a degree second only to tomatoes (FAO 2012). According to Brewster (2018), it is a short-duration horticultural crop.

Because of certain chemicals in the water, using wastewater to irrigate agricultural land damages plants' ability to divide mitotically, which ultimately leads to plant extinction (Caritá and Marin-Morales 2008). Eating these plants could have a negative impact on people's health. According to Sik et al. (2009), the chemical composition of plants cultivated in these fields may cause major side effects such as allergies at a young age, respiratory issues, heart problems, and cancer in middle age. Sewage wastes have been shown to be a dangerous factor since they have the potential to be genotoxic agents and can harm chromosomes, resulting in structural defects. The primary goal of this study is to evaluate how *A. cepa* roots develop in wastewater as opposed to tap water.

Material and methods

Onion preparation

For a period of 72 hours, little onion (*A. cepa*) bulbs of a similar size and weight (approximately 15-20g) were placed in beakers containing three sewage water samples, control water, and other water samples. The samples were gathered in from various parts of Koya City.

Experimental procedure

The onion bulbs were exposed for 72 hours in each experiment. They were placed in beakers with different water samples and stabilized with a wooden applicator. The experiment was completed after 72 hours, at which point the onion roots had grown.

Macroscopic parameters

The length of the root and additional characteristics, such as the typical shape and quantity of roots after 72 hours, are examples of macroscopic metrics.

Root and slide preparation

Three root tips from the actively growing plant were cut to be 1cm long using a scalpel or knives. Only the tapered end of the root tip was used. 2-3 roots were placed on a glass microscopic slide using forceps, and then covered with 2-3 drops of 1M hydrochloric acid. This helps to separate the tissue to improve visibility of the cells. After 5 minutes, any excess hydrochloric acid was blotted away using a paper towel. 2-3 drops of deionized water were then added to the root tips using a graduated pipet, and any excess water was blotted away with a paper towel. Next, 2% acetocarmine stain was added to the root tips using a pipette, and the root tips were soaked for approximately 3 minutes. Any excess acetocarmine was blotted away with a paper towel. One drop of deionized water was added to the root tips. Using forceps, one root tip was moved to a clean microscopic slide and a cover slip was placed on the root tissue. Gently applying pressure with a pencil, the root tissue was squashed to facilitate observation under the microscope and to observe the cell cycle phases.

Microscopic parameters

The scoring of dividing cells, the mitotic phases, and the mitotic index were all included in the cytogenetic study. The mitotic index, or MI, is the percentage ratio of the total number of scored cells to the number of dividing cells. The mitotic index drops as one gets farther away from the root tip. This indicates that as cells pass from the zone of cell division to the zone of cell elongation, their rate of division will gradually decrease. The rate at which cells divide inside a tissue is determined by the mitotic index. It is especially useful for assessing the effects of xenobiotics like pollutants, germination agents, and poisonous compounds. Accurately recognizing dividing cells is significant because cell division is a crucial process in the growth and repair of tissues in organisms. It also serves as an indicator of overall organismal growth. Careful microscopic inspection, especially in the cortex, can do this. Dividing cells are easily visible under a microscope since they are tiny and usually round in form. For a final conclusion

in situations where their division is not readily apparent, microscopic investigation is essential. Using a microscope guarantees accurate cell division identification. (Smail 2020, Ibrahim et al. 2021). The following calculations were used to compute the mitotic index and determine the proportion of each type of divided cell phase, such as prophase, metaphase, anaphase, and telophase, using slides observed under a compound microscope with an oil-immersed 100X objective lens.

$$\text{Mitotic index (\%)} = \frac{\text{Total number of dividing cell}}{\text{Total number of cell examined}} \times 100$$

2.6 Statistical analysis:

A one-way analysis of variance (ANOVA) test was used to examine the impact of sewage wastewater status on the phase index (%) of different wastewater sites of mitotic distributions in *A. cepa*. A statistically significant result is defined as a p-value less than 0.05. Additionally, the analysis was conducted using SPSS version 20.

Results

Table 1 shows how the mitotic index of *A. cepa* root tip cells is affected by sewage effluent from various places. In comparison to the groups exposed to wastewater, the control group, which was not exposed to wastewater, has a higher mitotic index (18%), indicating more active cell division. Significantly lower mitotic indices of 2.6%, 1.3%, and 2% at Azadi Bridge, Sar Razan, and Sar Baskan, respectively, imply that sewage wastewater has a cytotoxic effect that inhibits cell division.

Table 1. Effect of sewage wastewater on mitotic index on the root tip of *A. cepa*

Waster waste locations	Number of cell counted	Total number cell divided	Total number cell non divided
Control	150	27(18%)	123(82%)
Azadi Bridge	150	4(2.6%)	146(97.3%)
Sar Razan	150	2(1.3%)	148(98.6%)
Sar Baskan	150	3(2%)	147(98%)

The distribution of mitotic phases in *A. cepa* root cells exposed to sewage effluent from various places is displayed in the table. In the control group, the telophase has the highest frequency (44.4%), followed by prophase, metaphase and anaphase. On the other hand, groups exposed to wastewater have skewed distributions, particularly in cases where telophase percentages are elevated (e.g., Sar Razan at 50% and Azadi Bridge at 75%). The large F value (7.963) and P value (0.0035) support the idea that sewage effluent disturbs normal cell cycle development, resulting in notable variations in mitotic phase distribution (Table 2)

Table 2: Effect of sewage wastewater Status of phase index (%) of different wastewater locations of mitosis distributions *A. cepa*

Waste water locations	Prophase	Metaphase	Anaphase	Telophase	Total	F value	P value
Control	7(25.9%)	5(18.5%)	3(11.1%)	12(44.4%)	27(100%)	**7.963**	**0.0035**
Azadi Bridge	1(25%)	0(0%)	0(0%)	3(75%)	4(100%)		
Sar Razan	1(50%)	0(0%)	0(0%)	1(50%)	2(100%)		
Sar Baskan	2(66.6%)	0(0%)	0(0%)	1(33.3%)	3(100%)		

Significant = (P≤0.05), using One-Way ANOVA

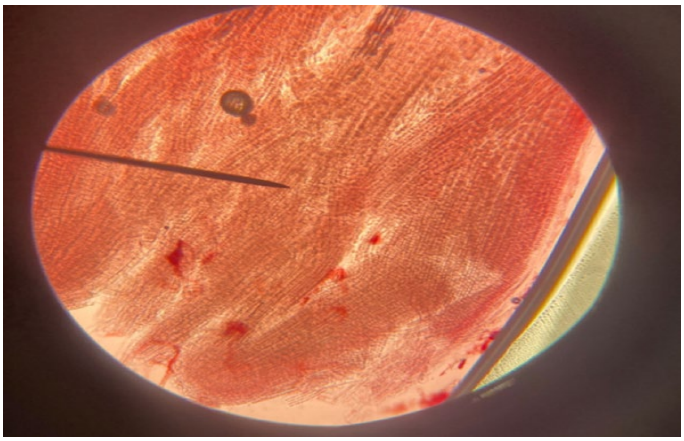


Figure 1. General aspect of the root meristem cells of *A. cepa* (40X magnification)

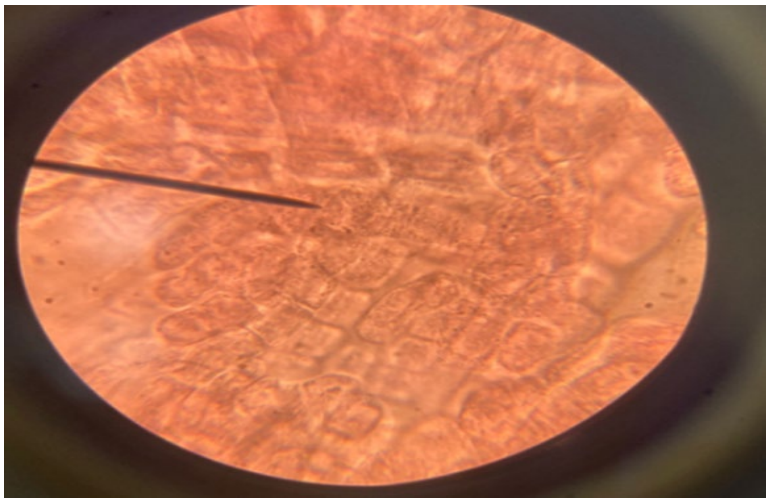


Figure 2. At a 100X magnification, meristematic cells of *Allium cepa* root tips in various phases are detected

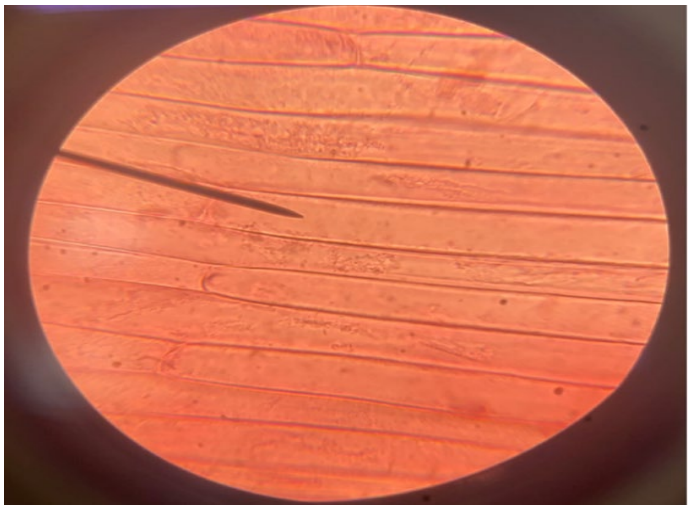


Figure 3. Meristematic cells at 100X magnification in the root tips of *A. cepa* are non-dividing cells in sewage effluent.

Discussions

In this experiment, the roots of *A. cepa* were grown in different water samples (control water, Azadi bridge, Sar Razan, and Sar Baskan) for a period of 72 hours. The growth rate differed in each sewage water sample compared to the control water sample. In each slide, 150 cells were counted. The growth rate and number of divided cells of the onion roots grown in the control water was 27 cells (18%). However, the number of undivided cells was 123 cells (82%). The number of divided cells in Azadi bridge water was 4 cells (2.6%), while the number of non-divided cells was 146 cells (97.3%). In Sar Razan's water, 2 cells (1.3%) were divided and 148 cells (98.6%) were undivided. The other sample, Sar Baskan, had 3 cells (2%) that divided, while the number of non-divided cells was 147 cells (98%) at 72 hours (Table 1).

Heavy metals are known as environmental pollutants that present a significant peril to living organisms. These substances are resistant to decomposition and tend to accumulate within organisms, leading to numerous detrimental effects. Many of the heavy metals, including mercury, lead, cadmium, chromium, zinc, copper, and nickel, are known to cause toxic effects (Alengebawy et al. 2021). The primary site for the uptake of these metals in plants is their root system. As heavy metals become adsorbed into the soil, they easily accumulate in plant roots (Shokri et al. 2022).

The escalating presence of heavy metal pollutants in water, soil, and air has emerged as a pressing issue in recent times. Even at minimal concentrations, heavy metal pollutants pose a considerable threat. Moreover, their non-biodegradability prevents them from breaking down into harmless forms. Consequently, heavy metals can accumulate in water sources and soil, subsequently being absorbed by plants. This poses a risk to plant life (Sharma et al. 2023). The presence of heavy metals and other contaminants in soil can cause similar detrimental effects, which can further result in the contamination of plants. The primary objective of this study is to gain a more comprehensive understanding of the consequences of sewage water waste and its impact on plants in terms of patterns, probability, and the extent of pollution when the *A. cepa* plant comes into contact with sewage-contaminated water (Goyal et al. 2020). *A. cepa* root growth is a fundamental endpoint for the investigation of heavy metals in contaminated water because root growth in *A. cepa* is believed to be suppressed by the presence of heavy metals, such as lead, in contaminated water. It can be used as an indicator of water toxicity (Lyu et al. 2020). Furthermore, *A. cepa* cells grow at a great rate due to their large size. This characteristic makes the species extremely susceptible to the impacts of heavy metals. Additionally, because *A. cepa* roots are large and have a much greater specific surface area than

other plant roots of similar masses, the roots and cells of onions are extremely susceptible to heavy metals. In order to develop efficient water treatment techniques (da Cunha Neto et al. 2023), meristematic cells of root tips of *A. cepa* were examined at 40X magnification, as shown in Figure 1.

The effect of sewage wastewater on the mitotic index of the examined root tips and the total number of examined cells (150 cells), classified into interphase and dividing cells (prophase, metaphase, anaphase, telophase), was shown in Table 2.

The percentage of mitotic index and number of divided cells in the control water (18%) are higher than in the other water samples. Lower mitotic index and divided cells (1.3%) were observed in the Sar Razans sewage water. The mitotic index and divided cells of other sewage water samples are significantly different in comparison with the control. The percentage of divided cells and mitotic index in Azadi bridges sewage water is 2.6% and in Sar Baskans sewage water is 2%, respectively. In the growth of *A. cepa* in control and different sewage water samples, there is a significant difference between the phases (prophase, metaphase, anaphase, telophase) (Table 2). Among the dividing cells, telophase has the highest number, followed by prophase, metaphase, and anaphase. The Azadi bridges sewage water has the highest percentage of telophase (75%), while Sar Baskans sewage water has the lowest percentage (33.3%). The other three stages dividing cells (prophase, metaphase, and anaphase) also differ. The control water and Azadi bridge sample have the same and lowest percentage of prophase (25.9%) and (25%) respectively, while Sar Baskans sample has the highest percentage of prophase (66.6%), followed by Sar Razans sample (50%). The lowest number of metaphase is seen in Azadi bridge, Sar Razan, and Sar Baskans samples, which are all 0%, followed by the control sample, which has the highest percentage (18.5%). The highest percentage of anaphase is seen in the control water sample (11.1%), but the lowest percentage of anaphase is seen in Azadi bridge, Sar Razan, and Sar Baskans samples, which are all 0% (Table 2). The cell division process is essentially a parent cell dividing into daughter cells. Apart from accomplishing simple multiplication of cells, it is also crucial for development and growth in multicellular organisms. Lastly, it is integral in regeneration and repair in multicellular organisms (Elchaninov et al. 2021).

A. cepa, a freshwater plant, holds great significance for multiple reasons. Firstly, it possesses such a productive biomass that it covers large areas of the substrate where it grows. Secondly, *Allium cepa* produces an abundant quantity of leaves, which it discards as a result of competition with other large aquatic plants or when environmental conditions are stable and suitable. The leaves of *A. cepa* are thin, broad, and short-lived, allowing them to efficiently capture light when it becomes available. Lastly, under favorable conditions, this plant can achieve a substantial biomass, only to begin decomposing swiftly, thereby adding a significant amount of organic matter to sediment and water (Paramonova et al. 2021).

Sewage water is a result of a combination of domestic, commercial, and industrial wastes. The water is produced by activities such as cleaning, through the use of detergents, bleaches, and soaps, cooking, through the boiling of water, and carrying out experiments in the laboratories that require the use of water. The major contributors to the production of sewage water are the commercial and industrial sectors (Gaur et al. 2020). Regardless, the water has been utilized and is no longer required, thus it is classified as either sewage water or wastewater.

Conclusions

To sum up, sewage-contaminated water may hinder the growth and division of *A. cepa* roots due to harmful heavy metals such as lead and mercury. The small size and surface area make *A. cepa* roots susceptible to toxicity caused by these metals. When compared with controls, mitotic index analysis shows that cell division is reduced in wastewater samples, while changes

in distinct phases during cell division emphasize diverse unfavorable impacts on root development triggered by contamination from sewage water.

References

Alengebawy A., Abdelkhalek S.T., Qureshi S.R., Wang M.Q. 2021. Heavy metals and pesticides toxicity in agricultural soil and plants: Ecological risks and human health implications. Toxics. 9(3): 42.

Brewster J.L. 2018. Physiology of crop growth and bulbing. In: *Onions and allied crops*. CRC press. p. 53-88

Caritá R., Marin-Morales M.A. 2008. Induction of chromosome aberrations in the Allium cepa test system caused by the exposure of seeds to industrial effluents contaminated with azo dyes. Chemosphere. 72(5): 722-725.

da Cunha Neto A.R., da Silva I.G., Calvelli J.V.B., Martins G.E.C., Carvalho M., Barbosa S. 2023. Toxicity of heavy metals that affect germination, development and cell cycle of *Allium cepa* L. Bulletin of Environmental Contamination and Toxicology. 111(2): 22.

Dahlgren R.M., Clifford H.T., Yeo P.F. 2012. The families of the monocotyledons: structure, evolution, and taxonomy. Springer Science & Business Media.

Darlington C.D. 1942. Chromosome chemistry and gene action. Nature. 149(3768): 66-69.

Elchaninov A., Sukhikh G., Fatkhudinov T. 2021. Evolution of regeneration in animals: A tangled story. Frontiers in Ecology and Evolution. 9: 621686.

Gaur V.K., Sharma P., Sirohi R., Awasthi M.K., Dussap C.G., Pandey A. 2020. Assessing the impact of industrial waste on environment and mitigation strategies: A comprehensive review. Journal of hazardous materials. 398: 123019.

Goyal D., Yadav A., Prasad M., Singh T.B., Shrivastav P., Ali A., ..., Mishra S. 2020. Effect of heavy metals on plant growth: an overview. Contaminants in agriculture: sources, impacts and management: 79-101.

Hanelt P. 2018. Taxonomy, evolution, and history. In: Onions and allied crops. CRC Press. p. 1-26.

Igwenyi I. 2012. Sewage management and its benefits to man. Vol. 3(10). p. 174-189.

FAO 2012. World onion production. Food and Agriculture Organization of the United Nations. http:// faostat.fao.org (accessed 27 February 2017).

Lyu G., Li D., Li S., Ning C., Qin R. 2020. Genotoxic effects and proteomic analysis on *Allium cepa* var. *agrogarum* L. root cells under Pb stress. Ecotoxicology. 29: 959-972.

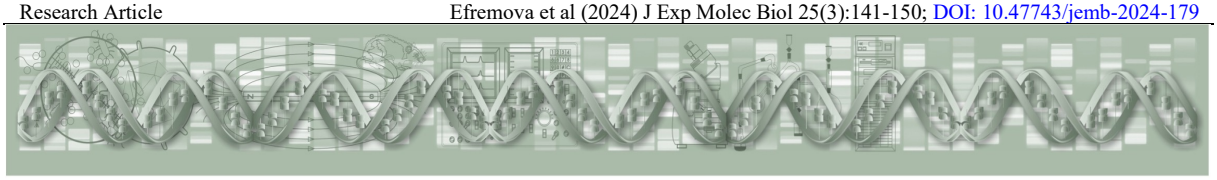
Paramonova T., Kuzmenkova N., Godyaeva M., Slominskaya E. 2021. Biometric traits of onion (*Allium cepa* L.) exposed to 137Cs and 243Am under hydroponic cultivation. Ecotoxicology and Environmental Safety. 207: 111191.

Rajasulochana P., Preethy V. 2016. Comparison on efficiency of various techniques in treatment of waste and sewage water–A comprehensive review. Resource-Efficient Technologies. 2(4): 175-184.

Sharma A., Grewal A.S., Sharma D., Srivastav A.L. 2023. Heavy metal contamination in water: consequences on human health and environment. In: Metals in water. Elsevier. p. 39-52.

Sik L., Acar O., Aki C. 2009. Genotoxic effects of industrial wastewater on *Allium cepa* L. African Journal of Biotechnology. 8(9).

Smail H.O. 2020. Impact of different concentration of Sodium chloride on the Root growth, Cell division and Chromosomal abnormalities in the root tips of *Allium cepa*. Journal of Advanced Laboratory Research in Biology. 11(1): 1-6.



BIOCHEMICAL CHARACTERIZATION OF THE YEAST BIOMASS RESULTING FROM THE WINEMAKING PROCESSES

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Abstract

Valorization of winemaking wasteis an actual ecological problem that needs to be solved. The current study was carried out to evaluate biochemical composition of *biomass* of *wine sedimentary yeasts*. According to obtained results,yeast sediments resulting in natural white and red wine making present a good source of protein as well, as of essential and immunoactive amino acids. The studied types of sediments possess high antioxidant activity and activity of antioxidant enzymes catalase and superoxid dismutase, the values varying, depending on the type of wine. So, yeast sediments of wine production can be proposed for the further development of new technologies for the production of bioactive extracts with antioxidants properties.
Keywords: *Saccharomyces cerevisiae*, vinification wastes, antioxidants, protein, enzyme

Introduction

During the last years winemaking has become the most dynamically developing branches of the Russian agro-industrial complex. According to a study, in 2022 world wine production amounted to 258 mhl, with a decrease of 3 mhl (-1%), compared to 2021. In Moldova vinified production is estimated at 1.4 mhl (OIV 2022). Waste recycling contributes to obtaining of valuable products needed for a large number of economical directions. Over the last decade, the intensive study of reutilization of wastes is important (Rodrigues 2014, Otles et al. 2015, Ericson 2022). Yeasts from wine production can serve as prime source for food and feed additives with high biological value.
Waste of wine production can be used for the production of natural cosmetics (Hiba et al. 2021). It is known that polyphenols - ingredients with high antioxidant properties are subsequently obtained from the extracts on the base of wine yeasts (Ky et al. 2014).
The wide variety of biochemical parameters of yeasts remained from wine production, might led to obtaining of the different bio preparations such as enzymatic, protein, mannoprotein preparations.
Disposal of production waste that pollutes the human environment is one of the most important environmental and economic problems of society. A lot of waste is generated during the production of wine. Complex processing of secondary raw materials of winemaking is recognized not only as necessary and useful from the point of view of environmental protection and recreational activities, as it helps to reduce environmental pollution, but also as a highly efficient type of commercial activity. The use of yeast as a source of bioactive substances and complexes is one of the important areas of modern biotechnology (Leon-Gonzales 2018). It is

Received 24 February 2024; Reviewed 10 July 2024; Accepted 29 July 2024;
Published Online 29 July 2024; Published 9 October 2024

known that yeasts can serve as a source of enzyme extracts that could be used also in food industry and save the value of food raw matter. According to recent studies, enzymes with pronounced antioxidant properties, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) have an important role in protection of live organisms from the negative consequences of free radicals and reactive oxygen species (Ighodaro and Akinloye 2018).

Bio-waste is defined as a result of technological food production. The development of technologies for the production of feed protein and other biologically active substances, based on waste from the wine industry, is relevant, both in terms of the safe use of this raw material and the elimination of environmental risks (Garcia-Lomillo et al. 2014, Iuga and Mironeasa 2020).Thus, the research goal was to evaluate biochemical composition of yeasts biomass obtained from the processes of wine production..

Wine production is one of the most important fields of food industry (Stopka et al. 2008). According to recent researchers, winery waste is characterized by a high concentration of organic substances, such as proteins, lipids, polyphenols, carbohydrates (Minusi et al. 2003, Zhang et al. 2017, Ferrer-Galego and Silva 2022).

Untreated it can harm the environment, including the soil, water, plants. Wine industry produces a large amount of wastewater and organic waste that must bere-used with the aim to avoid contaminating. Regrettably, only a few of these by-products are used for livestock sector and food industry. *Furthermore*, by-products of wine industry can be used for valorization of bioactive preparations, pharmaceutical, food, and cosmetic ingredients (Gurev et al. 2022). The new preparation can be used, also, in the fields of animal husbandry and veterinary medicine.

Currently, *scientific* researchers are aimed at improving the technology of wine production. Due to the increase of the wineryindustry, the problem of utilization of wine waste has arisen (Ye et al. 2015, Musteață et al. 2021). During the preparation of wine, sedimentary yeasts are formed, which contain a complex of organic compounds with biological value, so it is advisable to introduce environmentally safe and effective technologies for processing these types of waste, taking into account their physiological properties.

Thus, there are several solutions to increase the value of these numerous wastes, namely yeast biomass as a food additive and feed or source of biologically active substances, which, however, have not been widely used. Therefore, the alternative of utilization and recycling of these wastes is of great interest.

It is necessary to develop procedures for the recovery and transformation of by-products with a certain degree of innovation. It can therefore be difficult to reduce or minimize waste production in wine production processes that are limited by infrastructures or human resources. As a consequence, it is important to develop valorization procedures, leading to the further implementation of wastes of the wine industry.

Materials and Methods

The object of research were samples of wine yeast selected at a winery in «Cricova» SA. The yeast sediments from wine were subjected to centrifugation to separate the remaining liquid. The commercial *Saccharomyces cerevisiae* strains were used for winemaking. The yeasts biomass (*Saccharomyces cerevisiae*) of lower fermentation from the production of red *Merlot* (RSM –red sediments Merlot) and *Cabernet Sauvignon* (RSC- red sediments Cabernet) and white *Rkatsiteli* wine (WSR – white sediments Rkatsiteli) were offered by the wine complex.

The ABTS radical decolorization assay was used to evaluate *total antioxidant* activity of sedimentary yeasts samples (Re et. al.1999). The absorbance was measured at 734 nm.

% Inhibition = (Control Abs-Sample Abs)/Control Abs*100.

Control Abs – the absorbance of ABTS radical solution,

Sample Abs – the absorbance of ABTS radical solution +sample.

Folin Ciocalteau reagent has been used for the determination of protein content in samples of yeasts biomass from wine sediments (Lowry et al. 1951). The method is based on reactions of the colour complex formation. For the calibration curve was used bovine serum albumin as atandard. Based on the coefficient determined from the calibration curve, was used the calculation formula: C = A750·K·η·100/m,, where C - protein content in biomass (%); A_{750} - absorbance at 750 nm; K - recalculation coefficient (in mg protein/ml sample) determined from the calibration curve; η - dilution; 100 - % coefficient; m - mass of test sample, mg.

The total carbohydrate content was measured spectrophotometrically by the anthrone method. *Anthrone method* is one of the most used *methods* for the *determination* of soluble *carbohydrates* (Dey and Harborn 1993*). Carbohydrates estimation is measured at* wavelength of 620 nm. Carbohydrates are dehydrated at the reaction with concentrated H_2SO_4 to form furfural- a blue-green complex. A calibration curve was constructed in successive dilutions of the glucose standard solution. According to hydrolysis with anthrone and cooling of the samples to room temperature, the absorbance was measured at a wavelength of 620 nm relative to the control sample. Using the QUANTITATIVE WORKSPACE application provided with spectrophotometer, the value for the coefficient K used in the quantitative calculation of carbohydrates was obtained. The calculation formula is K=C/A620, where C -concentration of glucose in samples, mg/ml, A - absorbance of the sample at 620 nm.

The superoxide dismutase activity was determined as the inhibition or reduction of Nitroblue tetrazolium (NBT) in the presence of *Tetramethylethylenediamine* (TEMED) (Titova and Subbotina 2012). Measurements were conducted at a wavelength of 560 nm. The presence of the reaction medium blue tetetrazolium allows to estimate the decrease in superoxide radical production per unit based on photometric detection of the formation of diformazane (a product of reduction of nitroblue tetrazolium by superoxide radicals). SOD activity was determined using the following formula:

SOD activity (Units/mg protein) = ((Control Abs – Sample Abs)*100%)/C

Control Abs – the absorbance of control probe,

Sample Abs – the absorbance of sample probe,

C – concentration of protein (mg/l).

The catalase activity was measured according to the spectrophotometric assay that was *based* on the *ability* of *hydrogen peroxide* to *form salts* with *molybdenum stable* colored *complex* (Komina et al. 2012). The absorbance was registered on spectrophotometer through the changes of optical density against blank at 410 nm. Catalase activity was determined by the following formula:

CAT activity = (Control Abs-Sample Abs)*V/v • t • C, where

Control Abs – absorbance of a blank probe,

Sample Abs – absorbance of a sample probe,

V – total volume of the probe,

v – sample volume,

t – incubation time,

C – extinction coefficient (22.2*103 $mmol^{-1}*cm^{-1}$).

The determination of amino acids content was assessed by ion-exchange chromatography method is reliable for determining of the free amino acid content of different types of yeasts biomass from wine yeasts. For the determination of the composition of amino acid 100 mg of the yeast biomass were dried at 60 °C and were subjected to hydrolysis at 120 °C for 15 min with concentrated hydrochloric acid. The sample was filtered and 0,5 ml of the liquid was adjusted to volume of 2 ml with buffer solution, Ph = 2,2 and was subjected to analysis. The amino acid analyzer AAA-339M (CZECH Republic) was used for the analysis (Garaeva et al. 2009).

Quantitative determination of macro and micro elements and heavy metals accumulated in yeasts biomass was evaluated by ICP-OES method. The digestion was carried out by the following procedure: 2.0 ml concentrated HNO_3 and 1.0 ml H_2O_2 was added to 50 mg of yeast biomass (sample). The digestion was carried out by the autoclave method. After digestion the samples were transferred to 50 ml volumetric flasks, and deionised water was added for the final volume. The macro and microelements s were determined by ICP-OES. The content of macro elements, microelements and heavy metals was determined by the spectrophotometric method, at Thermo Scientific iCAP 6200 Duo spectrometer Scientific, United Kingdom (U.S. EPA. 2007).

Ash content was determined by weight of inorganic matter remaining after the removing water and organic matter (Harris, Marshall 2017). The ash content was gravimetrically determined by muffle furnace ignition at 550° C.

The lipid content was determined gravimetrically by the method proposed by Bligh and Dyer (Bligh 1959). For the lipid extraction to 1,0 g of yeast biomass the mixture 1:2:0,8 (v/v/v) cloroform –ethanol – water was used. Extraction is carried out at room temperature by continuous stirring for 60 min. Chloroform is removed by distillation in a vacuum evaporator at 40^0C. The lipid mass obtained is dried at temperature 105 ± 20C to a stable mass, for which at least 3 times. Using the ratio of the amount of lipid obtained to the biomass used for extraction is determined is determined by the following formula.

Lipids content, $\%=m_{Lipids}*100/m_{sample,}$ where

m_{lipids} – dry weight of lipids obtained by the extraction procedure,

m_{sample} -Dry weight of yeast biomass sample.

Statystical analysis. The obtained data were analyzed using one-way Analysis of variance (ANOVA). The mean and standard deviation was calculated to demonstrate quantative variables. P – values less than 0.05 were considered significant.

Results and discussion

Because, in order to evaluate the prospect of use and the spectrum of possible products, it is necessary to know the biochemical composition of the yeast biomass. At the initial stage the total content of proteins (including amino acids), carbohydrates, lipids, macro-, micro-, trace elements, heavy metals, total antioxidant activity and activity of antioxidant enzyme such as catalase and superoxide dismutase activity was studied in the three types of yeast biomass. These types were obtained from red, white wine production, as well as red wine production with perlite.

The results of the study of biochemical composition are presented below (Figure 1).

It can be mentioned that yeast biomass from the production of red wine Merlot and Cabernet contains significant quantities of proteins 65.97±0.45 and 64.74%±0.27% per yeast dry weight and have the minimum amount of lipides 2.47±0.03 and 4.22±0.14 % per yeast dry weight, respectively. While yeast biomass from the production of white wine *Rkatsiteli* contains proteins - 32.0 % per yeast dry weight and 11.00±1.0 %. per yeast dry weight. The maximum content of carbohydrates was found in the yeast biomass RSM. Thus, the biochemical analysis has demonstrated significant differences in the total protein content and lipid content among the studied wine wastes.

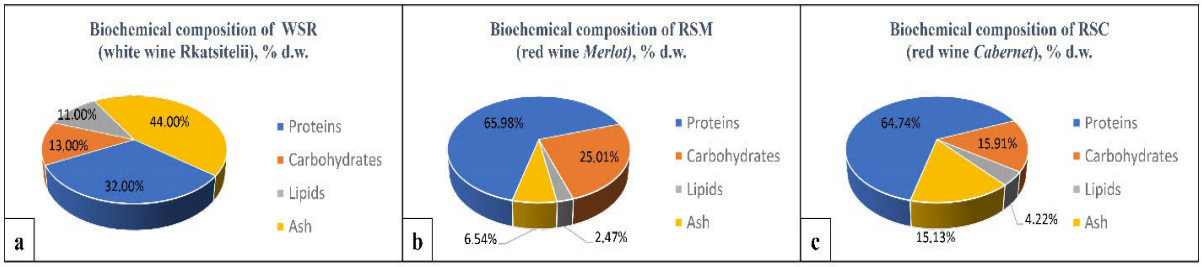


Figure 1. Biochemical composition of yeast biomass from the wine waste, WSR – **a)**, RSM - **b**, RSC – **c)**

In addition to the fact that wine yeasts contain significant amounts of proteins and biologically active polysaccharides, they are used a source of antioxidant substances. For these reasons, total antioxidant activity and the activity of antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) were further evaluated in the wastes of wine production (Table 1).
Thus, it has been established that all sediments possess antioxidant activity, but the values vary from case to case, depending on the type of wine. *Antioxidant activity* was measured by *ABTS* radical scavenging *assay.* For example, RSM has highest total antioxidant activity equivalent to 61.5±2.0% inhibition, while CAT activity is the lowest of all and constitutes 222.04±10.28 mmol/min. per mg protein. The total antioxidant activity of WSR and RSC is lower than that of SRM and constitutes respectively 47.2±3.8 and 49.9±0.3% inhibition, while CAT activity in both cases is high - 859.61±38.05 and 739.23±5.51 mmol/min. mg protein respectively. SOD activity, in all three sediment types, is comparable and constitutes from 171.0±5.8 to 227.0±2.52 U/mg protein.

Table 1. Antioxidant activity and activity of CAT, SOD enzymes in yeast biomass from the production of dry white and red wines from the wine complex "Cricova" SA.

Indices	**WSR**	**RSM**	**RSC**
Catalase activity (CAT), mmol/min. mg protein	859.61±22.0	222.04±5.9	739.23±3.18
Superoxid dismutase activity (SOD),U/mg protein	171.0±5.8	227.0±1.5	211.0±5.5
ABTS, % inhibition	47.2±3.8	61.5±2.0	49.9±0.3

Amino acids are used as active ingredients in the production of food supplements or in the pharmaceutical industry. The study of the amino acid content of yeast biomass from the wine waste has revealed that the sediments from white and red winemaking contain the full range of proteinogenic amino acids(cysteine, aspartic acid, threonine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, arginine), but their content differs depending on the type of wine (Figure 2).

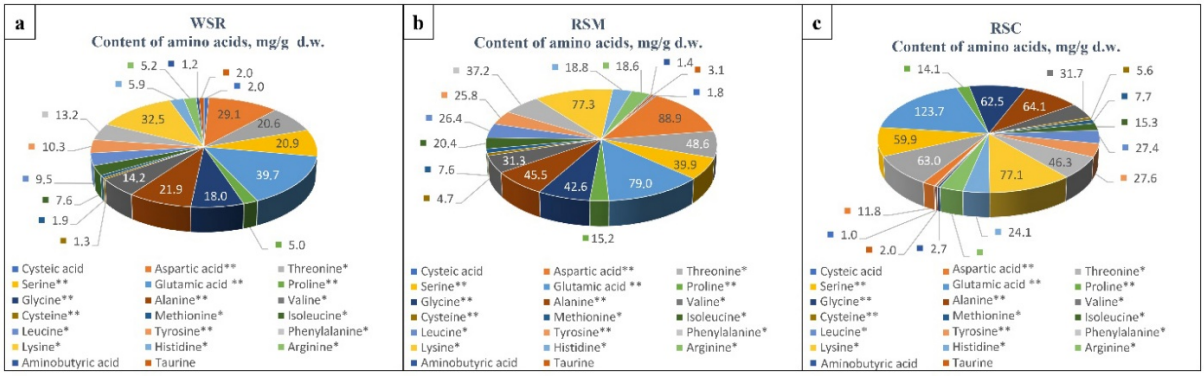


Figure 2. Amino acid content of yeasts biomass from the wine waste, WSR **(a)**, red RSM **(b),** RSC **(c)**

Thus, the sum of amino acids detected in WSR - 256.61 mg/g dry weight was lower, compared to the same indices of RSM and RSC - 627.52 and 683.64 mg/g dry weight. Considerable amounts of essential amino acids, such as phenylalanine, histidine, isoleucine were determined in yeast biomass of the studied variants. Similar results have been reported previously according to which, high content of histidine and phenylalanine was revealed in wine by-products (Buzzanka et al. 2024) and wine samples fermented with yeast strains (Taran and Antohi 2014). It is necessary to mentione the high content of essential and immunoactive amino acids in red wine sediments, the sum of which was 286.1-314.45 and 400.33-446.83 mg/g dry weight, respectively, that indicates the biological and nutritional value of wine yeast biomass (Figure 3).

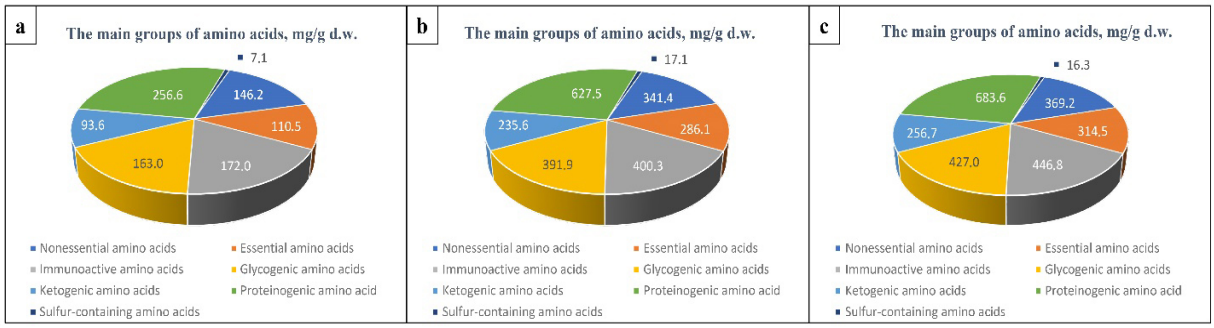


Figure 3. The main groups of amino acids of yeasts biomass from the wine waste, WSR – **(a)**, RSM – **(b),** RSC – **(c)**

The study has assessed the *content of macro*, micro, and heavy metals elements in wine sediments from the production of dry white and red wines (Figure 4-5).

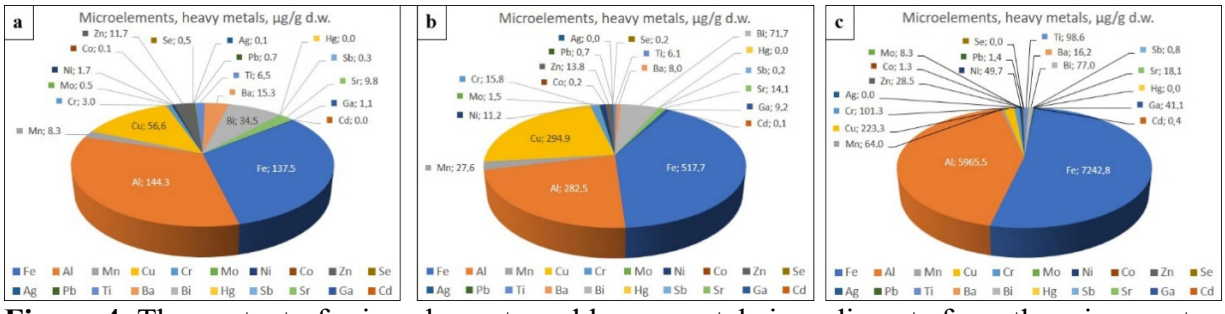


Figure 4. The content of microelements and heavy metals in sediments from the wine waste, WSR – **(a)**, RSM – (**b),** RSC – **(c)** from the wine complex «Cricova» SA

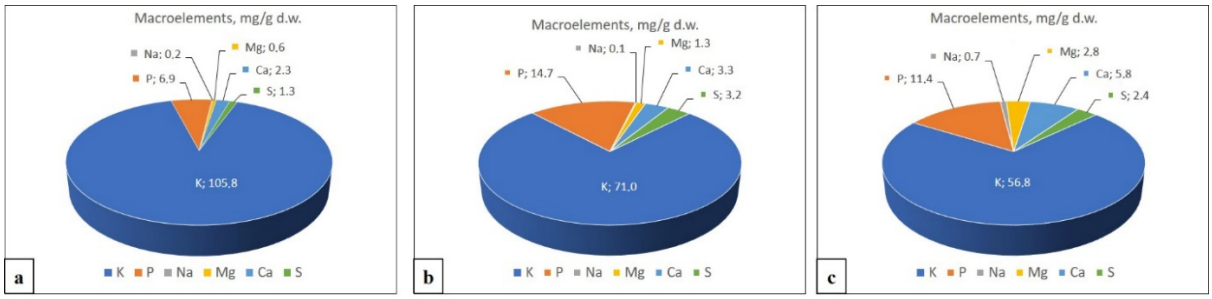


Figure 5. The content of macroelements in sediments from the production of dry white and red wines, WSR – **(a),** RSM – **(b),** RSC – **(c)** from the wine complex «Cricova» SA

Thus, it was established that yeasts sediments contain macroelements: K 56.8-105.8 mg/g dry weight; P 6.9-14.7 mg/g dry weight; Na 0.1-0.7 mg/g dry weight; Mg 0.6-2.8 mg/g dry weight; Ca 2.3-5.8 mg/g dry weight; S 1.3-3.2 mg/g dry weight. It should be mentioned the very wide spectrum of trace elements in the yeasts from wine sediments: Fe; Al; Mn; With; Cr; Mo; Us; Co; Zn; Se; Ag; Li; V; B; Rb in, the concentration of which varies within very wide limits. According to the literature data, yeast strains might affect the content of Co, Cu, Mg, Na, Pb, Sr and Zn in the wine. (Nicolini 2003).
According to the obtained results, yeast sediments from white and red winemaking might serve as source of protein, the nutritional value of which is expressed by the high content of essential and immunoactive amino acids. Wine sediment yeasts can be used for the development of nutritional supplements and feed additives.

Conclusions

Given the increased interest in wine-making, there is an increasing necessity to reduce wine wastes through the development of new technologies for the production of bioactive extracts on the base of wine making yeasts. Yeast sediments used in natural white and red winemaking represent a good source of protein, the nutritional value of which is expressed by the high content of essential and immunoactive amino acids, a source of lipids, macro- and microelements polysaccharides, also, possess high antioxidant activity and activity CAT and SOD enzymes and can serve as a basis for the development of food supplements, feed additives and biologically active antioxidant preparations for increasing of the reproductive potential of livestock animals.Yeast preparations derived from sedimented wine yeasts may be used in the animal husbandry, food and cosmetic industries.The use of winemaking industry by-products could reduce the environmental impact and elaborate a promising way of its application in different areas of modern economy.

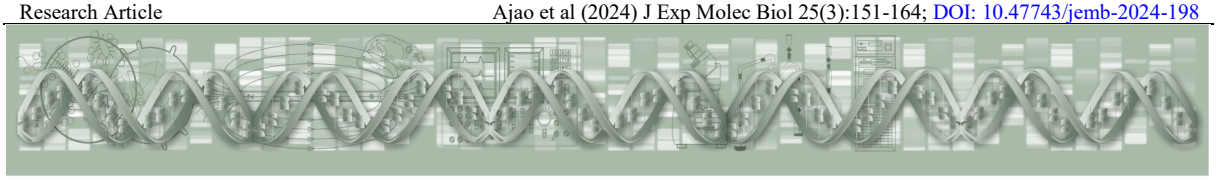
Acknowledgments: The results were obtained within the Project 20.80009.5107.16. funded by the NARD of Moldova

References

Bligh E, Dyer W. 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology. 37(8): 911-917.
Buzzanka C, Mauro M, Vazzana M, A Todaro, Arizza V, Lucarini M, Durazzo A, Vita Di Stefano V. 2024. Analysis of the amino acid profile of red and white graphs winery by-products from western Sicily. Measurement: Food. 14:100174.
Dey P, Harborn J. 1993. Methods in Plant Biochemistry. Carbohydr. Academic Press. 2.

Eriksson H. 2022. Organic waste streams in the wine industry. Independent Project in Food Science. Uppsala. Swedish University of Agricultural Sciences.
Ferrer-Gallego R, Silva P. 2022. The wine industry by-products: applications for food industry and health benefits. Antioxidants. 11(10):1-21. https://doi.org/10.3390/antiox11102025.
Garaeva SN, Redkozubova GV, Postolati GV. 2009. Aminokislotî v jîvom organizme. 2009. Academy of Science of Moldova. Institute of physiology and sanocreatology.
García-Lomillo J, González-Sanjosé ML, Del Pino-García R, Rivero-Pérez, MD, Muñiz-Rodríguez P. ss2014. Antioxidant and antimicrobial properties of wine byproducts and their potential uses in the food industry. J. Agric. Food Chem. 62:12595–12602.https://doi.org/10.1021/jf5042678.
Gurev A, Dragancea V, Baerle A, Netreba N, Boestean O, Haritonov S, Gaina B. 2022. Properties of winemaking by-products of feteasca neagra grape seeds. Chemistry journal of Moldova. 17(2):50-61. http://dx.doi.org/10.19261/cjm.2022.946.
Harris GK, Marshall MR. Ash Analysis. 2017.Food Analysis: 287–297.https://doi.org/10.1007/978-3-319-45776-5_16.
Hiba IHN, Rindala R, Khoury E, Youssef S, Letizia M, Manconi MM. 2021. Valorization of Wine-Making By-Products' Extracts in Cosmetics. Cosmetics 8(4):109.https://doi.org/10.3390/cosmetics8040109.
Ighodaro OM, Akinloye OA. 2018. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alexandria Journal of Medicine. 54(4):287-293.https://doi.org/10.1016/j.ajme.2017.09.001.
Iuga M, Mironeasa S. 2020. Potential of grape byproducts as functional ingredients in baked goods and pasta. Compr. Rev. Food Sci. Food Saf. 19(5):2473–2505. https://doi.org/10.1111/1541-4337.12597.
Komina AV, Korostileva KA, Gyrylova SN, Belonogov RN, Ruksha TG. 2012. Interaction Between Single Nucleotide Polymorphism in Catalase Gene and Catalase Activity Under the Conditions of Oxidative Stress. Physiol. Res. 61: 655-658. https://doi.org/10.33549/physiolres.932333.
Ky I, Crozier A, Cros G, Teissedre P. 2014. Polyphenols composition of wine and grape sub-products and potential effects on chronic diseases. Nutr. Aging. 2: 165–177. https://doi.org/10.3233/NUA-130027.
León-González ME, Gómez-Mejía E, Rosales-Conrado N, Madrid-Albarrán Y. 2018. Residual brewing yeast as a source of polyphenols: Extraction, identification and quantification by chromatographic and chemometric tools. Food Chem. 267:246-254. https://doi.org/10.1016/j.scitotenv.2017.06.141.
Lowry O, Rosebrough N, Farr A, Randall R. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
Minussi RC, Rossi M, Bologna L, Cordi L, Rotilio D, Pastore GM, Durán N. 2003. Phenolic compounds and total antioxidant potential of commercial wines. Food Chemistry. 82(3): 409–416. https://doi.org/10.1016/S0308-8146(02)00590-3
Musteață G, Balanuță A, Reșitca V, Razvan V, Băetu M, Patraș A. 2021. Capitalization of secondary wine products - an opportunity for the wine sector of republic of Moldova and Romania. Journal of Social Sciences. 4(2):117–127. https://doi.org/10.52326/jss.utm.2021.4(2).12.
Nicolini G, Larcher R. 2003. Evidence of changes in the micro-element composition of wine due to the yeast strain. 56(4):45-48.
OIV - International Organisation of Vine and Wine. 2022. STATE OF THE WORLD VINE AND WINE SECTOR IN 2022.

Otles S, Despoudi S, Bucatariu C, Kartal C. 2015 Food Waste Management, Valorization, and Sustainability in the Food Industry. Galanakis CM Ed. Food Waste Recovery, Elsevier, Amsterdam. p. 3-23. https://doi.org/10.1016/B978-0-12-800351-0.00001-8.
Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology & Medicine.26 (9-10): 1231-1237. https://doi.org/10.1016/S0891-5849(98)00315-3
Stopka P, Křížová J,Vrchotová N, Bábíková P, Tříska J, Balík J, Kyseláková M. 2008. Antioxidant Activity of Wines and Related Matters Studied by EPR Spectroscopy. Czech Journal of Food Sciences. 26(10):49-54. https://doi.org/10.17221/248/2008-CJFSSW-846.
U.S. EPA. 2007. "Method 3015A (SW-846): Microwave Assisted Acid Digestion of Aqueous Samples and Extracts," Revision 1. Washington, DC.
Taran N, Antohi M. 2014. Influence of the yeasts and enzyme preparations on content of amino acids in dry white wines. 2nd International Conference on Microbial Biotechnology. 79-83.
Titova N, Subbotina T. 2012. Enzymology: Laboratory Workshop. (in Russian). Krasnoyarsk: Siberian Federal University.
Ye Z, Harrison R, Cheng VJ, Bekhit A. 2015. Wine making by-products. M. Bordiga (Ed.). Valorization of wine making by-products. p. 73–115. CRC Press. https://doi.org/10.1201/b19423.Zhang N, Hoadley A, Patel J, Lim S, Li C. 2017. Sustainable options for the utilization of solid residues from wine production. Waste management (Elmsford). 60: 173–183. https://doi.org/10.1016/j.wasman.2017.01.006.



ENHANCING BIODEGRADATION EFFICIENCY OF REACTIVE BLACK-5 DYE USING *BACILLUS WIEDMANNII* STRAIN NAF4: OPTIMIZATION OF DEGRADATION PARAMETERS VIA RESPONSE SURFACE METHODOLOGY (RSM)

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Abstract

Reactive Black 5 (RB-5) dye is widely used in industries such as textile, paper, and leather, raising environmental concerns due to its persistence and adverse effects. This study aimed to develop efficient and eco-friendly strategies for RB-5 dye removal from industrial wastewater. RB-5 dye-degrading bacteria, namely NAF1, NAF2, NAF3, and NAF4, were isolated from soil contaminated with textile effluents. Evaluation of their decolorization potential revealed NAF4 as the most effective, achieving a decolorization percentage of 89%, followed by NAF3 and the co-culture at 75% and 73%, respectively. The isolate NAF4 showed significant production of tyrosinase and laccase enzymes. The 16SrRNA sequencing confirmed the identities of the isolates belonging to the following genera *Bacillus, Pseudomonas, Escherichia*, and *Citrobacter.* The biodegradation potential of B. wiedmannii strain NAF4 for RB-5 dye was assessed using Response Surface Methodology (RSM). The optimized conditions for RB-5 degradation were determined to be an agitation speed of 115.777 rpm, pH of 7.449, inoculum size of 12.255, and temperature of 29.74°C. The RSM model exhibited high statistical significance with an F-value of 53.30 and low p-values (<0.0001) as well as a correlation coefficient (R^2) value of 0.9813. Validation studies confirmed the adequacy and precision of the model. The maximum RB-5 degradation achieved was 90.2291%. This study provides insights into the potential applicability of RSM for optimizing degradation processes in various contexts and offers promising solutions for RB-5 dye removal from industrial wastewater, mitigating its environmental impact.
Keywords: *Bacillus wiedmannii*, Response Surface Methodology (RSM), Reactive Black 5, biodegradation, environmental impact, industrial wastewater

Introduction

The textile industry plays a significant role in the global economy, providing millions of jobs and meeting the growing demands for clothing and textiles (Dixit and Lal 2019). However, the production of textiles is associated with various environmental concerns, particularly the discharge of dye-containing wastewater (Al-Tohamy et al. 2022, Khan et al. 2023)
Reactive Black 5 (RB5) dye, a widely used textile dye, poses a significant threat to the environment due to its persistence, toxicity, and non-biodegradability (Elgarahy et al. 2021). The accumulation of RB5 dye in water bodies can result in severe ecological imbalances and harm aquatic life (Yusuf 2019).
Given the concerns associated with the persistence and adverse environmental effects of RB-5 dye, it is crucial to address the issue and develop effective strategies for its removal and

Received 21 May 2024; Reviewed 2 July 2024; Accepted 29 July 2024;
Published Online 29 July 2024; Published 9 October 2024

degradation. The development of sustainable and environmentally friendly methods for the treatment of RB-5 dye-contaminated wastewater is of utmost importance. Exploring and enhancing the potential of biological approaches, such as microbial degradation, it may be possible to mitigate the adverse environmental impacts associated with RB-5 dye.

Biodegradation has emerged as a promising approach for the removal of synthetic dyes from wastewater, offering a cost-effective and environmentally friendly solution (Srivastava et al. 2022). A wide range of microorganisms have been identified as potential sources of enzymes capable of degrading complex organic compounds, including synthetic dyes (Gaur et al. 2018). Consequently, there is a pressing requirement to explore and establish efficient and sustainable approaches for the removal and degradation of RB-5 dye from industrial wastewater.

This study represents a noteworthy advancement as it seeks to enhance current understanding by investigating the capabilities of biological mechanisms, specifically microbial degradation, in tackling the challenges associated with RB-5 dye contamination. The main goal of this research is to devise effective and environmentally friendly approaches for mitigating the persistent and detrimental environmental impact caused by RB-5 dye in industrial wastewater. Additionally, the efficacy of utilizing Response Surface Methodology (RSM) as an optimization tool for maximizing the degradation process by fine-tuning the degradation parameters has been demonstrated.

This study evaluates the biodegradation potential of *Bacillus wiedmannii strain NAF4* for Reactive Black 5 dye using a Surface Response Optimization (SRO) model. The SRO model will be employed to optimize the process parameters and determine the optimal conditions for maximum dye degradation efficiency. By exploring the biodegradation capabilities of *B. wiedmannii* and optimizing the process parameters using the SRO model, this study aims to contribute to the development of effective and eco-friendly solutions for the removal of RB5 dye from textile wastewater.

Materials and Methods

Chemicals. RB5 was procured from the local market in Ilorin. The structure of RB-5 is shown in Figure 1.

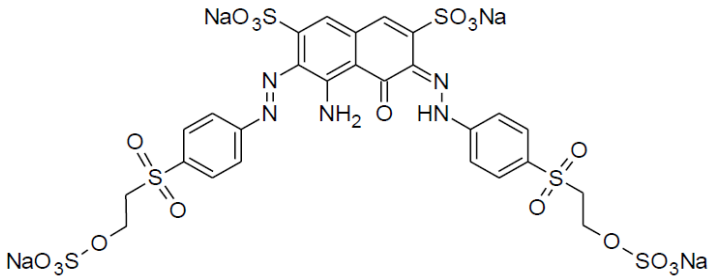


Fig. 1: Chemical structure of the dye "Reactive Black 5 (Pérez García et al. 2017)

Sample Area and sample Collection

The study was conducted at a local textile outlet situated in Adewole housing estate, Ilorin, Kwara state. This facility was chosen due to its practice of discharging effluent directly into the surrounding soil. To ensure a significant presence of dye effluent contamination, a thorough examination of the site was performed. Soil samples were collected during each phase of the study using aseptic techniques. The topsoil (0 to 5 cm) was carefully removed, and approximately 500 g of soil was obtained from various points. The collected soil samples were immediately transferred into a universal bottle using a soil auger (Chikere and Ekwuabu 2014). To maintain sample integrity, the universal bottle was securely packaged in a polythene bag and transported to the laboratory for further analysis.

Enrichment and Isolation of Dye-degrading Bacteria

The procedure described by Khan and Malik (2018) for isolating dye degrading bacteria utilized an enrichment technique based on the methodology outlined by Moosvi et al. (2005) with certain modifications. To initiate the isolation process, nutrient broth supplemented with RB5 at a concentration of 100 µg mL^{-1} was prepared. A 10% (w/v) portion of contaminated soil was inoculated into the broth, and the mixture was incubated under static conditions at 37 °C for 48 hours. This step was repeated several times using fresh dye-containing media until decolorization of the media was observed. To obtain pure cultures, a 100 µL sample of the culture suspension was plated onto nutrient agar plates containing RB5 at a concentration of 100 µg mL^{-1}. Following incubation, a bacterial colony showing the largest clear zone was selected for purification. The purified colony was then subjected to identification through 16S rDNA sequencing.

Biodegradation of RB5 by bacterial Isolates

The experimental setup involved utilizing a 250 ml conical flask containing 100 ml of nutrient broth as the growth medium. The broth was supplemented with Reactive Black 5 dye at a concentration of 10 µg/mL^{-1}. The basal conditions included a pH value of 7.0, a temperature of 37°C, and agitation at 150 rpm. For inoculation, a log growth phase culture of the target bacterium was prepared using the same nutrient medium conditions. The optical density (OD) of the inoculum was adjusted to 0.1 at a wavelength of 620 nm, corresponding to a bacterial concentration of 1×10^8 colony-forming units (cfu per ml), based on the McFarland turbidity standard of 0.5. Samples were periodically taken from the cultured broth and determined the percentage degradation.

Molecular Identification of the Dye-Degrading Bacteria

A single colony of respective dye-degrading bacteria was suspended in lysis buffer and Proteinase K for the extraction of the genomic DNA as described by Bhutia et al. (2021). The dye-degrading bacteria that were isolated had their 16S rRNA genes amplified through the use of universal primers 518F (5-CCAGCAGCCGTAATACG-3) and 800R (5-TACCAGGGTATCTAATCC-3) in a thermal cycler. The thermal cycling conditions included denaturation (92°C/min), annealing (54°C/min), and extension (72°C/min) for 25 cycles. The resulting PCR product was then assessed on 1% (w/v) agarose gels and purified using the QIA quick PCR purification kit from Qiagen, USA. The 16S rRNA genes were partially sequenced with the aid of the BIG-DYE terminator kit ABI 310 Genetic Analyzer from Applied Biosystems, USA. To align the bacterial sequences, the BLAST Search software at the National Center for Biotechnology Information was utilized to find homologous bacteria, the sequences. Neighboring Joining method was used to build the phylogenetic tree using MEGA 6. Upon submission to the National Center for Biotechnology Information (NCBI) gene bank, accessio numbers were assigned to the bacterial sequences.

Experimental Procedure

The experiments were carried out according to the Box–Behnken design and the range and level of the variables are stated in Table 1. The Box-Behnken design (BBD) of the response surface methodology (RSM) was used to optimize the experimental conditions vis-a-vis: temperature (20, 30, and 40 ^{0}C), pH (5, 7, 9), agitation (100,150, 200 rpm) and inoculum size for the degradation of reactive black-5 dye RB-5 by *B. wiedmannii*. The degradation capacity of *B. wiedmannii* was examined by inoculating the *B. wiedmannii* culture into different Erlenmeyer flasks (250 mL) containing minimal salt medium (50 mL) with above mentioned optimized conditions and incubated in an incubator shaker with the agitation speed and temperature adjusted according to the BBD After the degradation process, the samples were withdrawn and analyzed for RB-5.

The percentage degradation was calculated by

$$\text{Decolorization (\%)} = \frac{\textit{Initial OD} - \textit{Final OD}}{\textit{Initial OD}} \times 100\%$$

Experimental design by RSM

Response Surface methodology (RSM) is an empirical statistical technique employed for multiple regression analysis by using quantitative data obtained from properly designed experiments to solve multivariate equations simultaneously. Box–Behnken design was used to study the effects of the variables toward their responses and subsequently in the optimization studies. This method was suitable for fitting a quadratic surface and it helps to optimize the effective parameters with a minimum number of experiments, as well as to analyze the interaction between the parameters. The coded values of the process parameters were presented in Table 1. The experimental and predicted values of percentage degradation of RB5 by dye-degrading *B. wiedmannii* NAF4 are given in Table 2.

The regression and graphical analysis with statistical significance were carried out using Design Expert software (version 7.1.5, Stat-Ease, Inc., Minneapolis, USA). In order to visualize the relationship between the experimental variables and responses, 3D plots were generated from the models. The optimum values of the process variables are obtained from the response surface.

Table 1. Level of different process variables in coded and uncoded form for degradation of RB5 by *Bacillus wiedmannii* NAF4

Variables	Code	Levels		
		-1	0	+1
pH	A	5	7	9
Temperature, ^{0}C	B	20	30	40
Innoculum concentration, %	C	5	10	15
Agitation speed, rpm	D	100	150	200

Table 2. Experimental conditions of Box Behnken design for RB5 degradation by *B. wiedmannii* NAF4

		Factor 1	Factor 2	Factor 3	Factor 4	Response 1
Std	Run	A:Temp	B:pH	C:Inoculum size	D:Agitation	Degradation
		ºC		%		%
18	1	45	7	5	150	61
23	2	35	3	15	250	0
26	3	35	7	15	150	71
9	4	25	7	15	50	57
5	5	35	7	5	50	43
24	6	35	11	15	250	51
2	7	45	3	15	150	0
22	8	35	11	15	50	37
25	9	35	7	15	150	71
27	10	35	7	15	150	71
20	11	45	7	25	150	78

21	12	35	3	15	50	0
6	13	35	7	25	50	45
28	14	35	7	15	150	71
3	15	25	11	15	150	55
17	16	25	7	5	150	61
16	17	35	11	25	150	65
1	18	25	3	15	150	0
4	19	45	11	15	150	55
13	20	35	3	5	150	0
14	21	35	11	5	150	77
15	22	35	3	25	150	0
8	23	35	7	25	250	93
10	24	45	7	15	50	62
11	25	25	7	15	250	86
7	26	35	7	5	250	66
19	27	25	7	25	150	78
12	28	45	7	15	250	86
29	29	35	7	15	150	71

Results

The laboratory evaluation of their decolorization potentials showed variations in the abilities of four isolates, namely *C. freundi* NAF1, *E. coli* strain NAF2, *P. aeruginosa* strain NAF3, and *B. wiedmannii* NAF4, to degrade RB-5 dye. Among these isolates, NAF4 exhibited the highest percentage of dye decolorization, indicating its strong potential for degrading the dye. *P. aeruginosa* strain NAF3 showed the second highest decolorization ability, more than their co-culture while NAF2 and NAF1 demonstrated the lowest potential for decolorization as shown in Figure 1. The highest percentage of decolorization for all isolates was observed at 144 hours of incubation, indicating that the degradation process reached its peak at this time point. Subsequently, the percentage of decolorization gradually decreased over time.

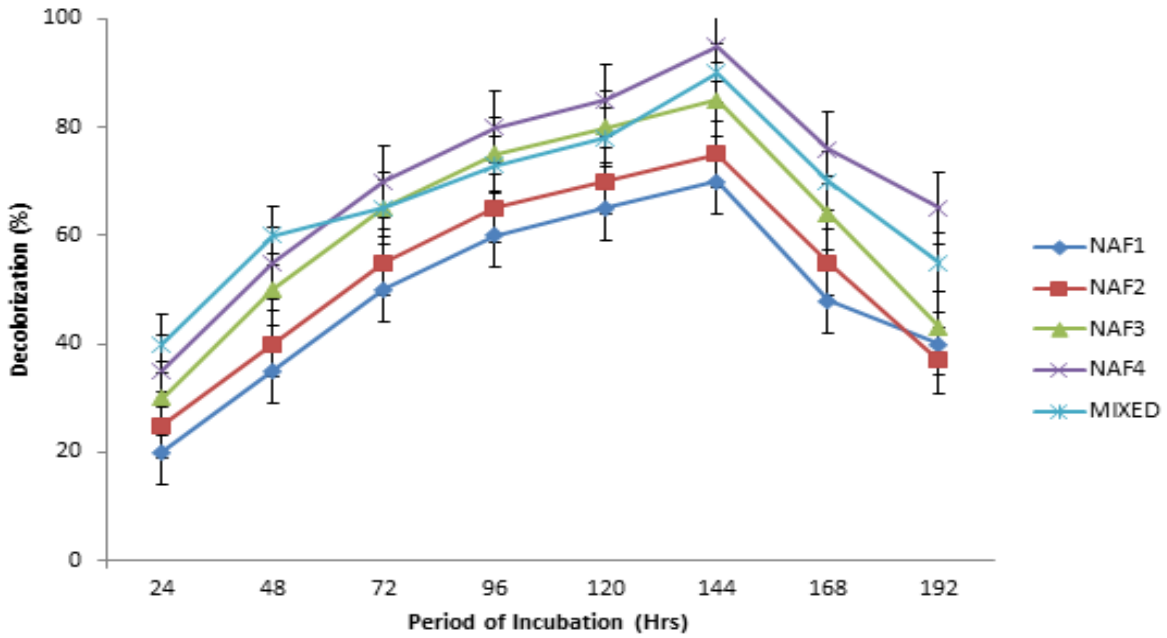


Figure 1. Decolorization of RB-5 by Bacterial Isolates from the Soil Contaminated with Textile Effluent

In this study, a total of four bacterial isolates were recovered from the soil samples contaminated with the dyes using culture-based methods. The dye-degrading bacteria belong to the following genera: *E.coli*, *Citrobacter, Pseudomonas and Bacillus.* The 16SrDNA sequencing revealed that the isolated bacterial strains exhibited homology to known species. Specifically, NAF1 to *Escherichia coli* strain 81402; NAF2, *Citrobacter freundii* strain ATCC 8090; NAF3 *Pseudomonas aeruginosa* strain DSM 50071 and NAF4, *Bacillus wiedmannii* strain BCL-Z09 was observed, as shown in Figure 2.

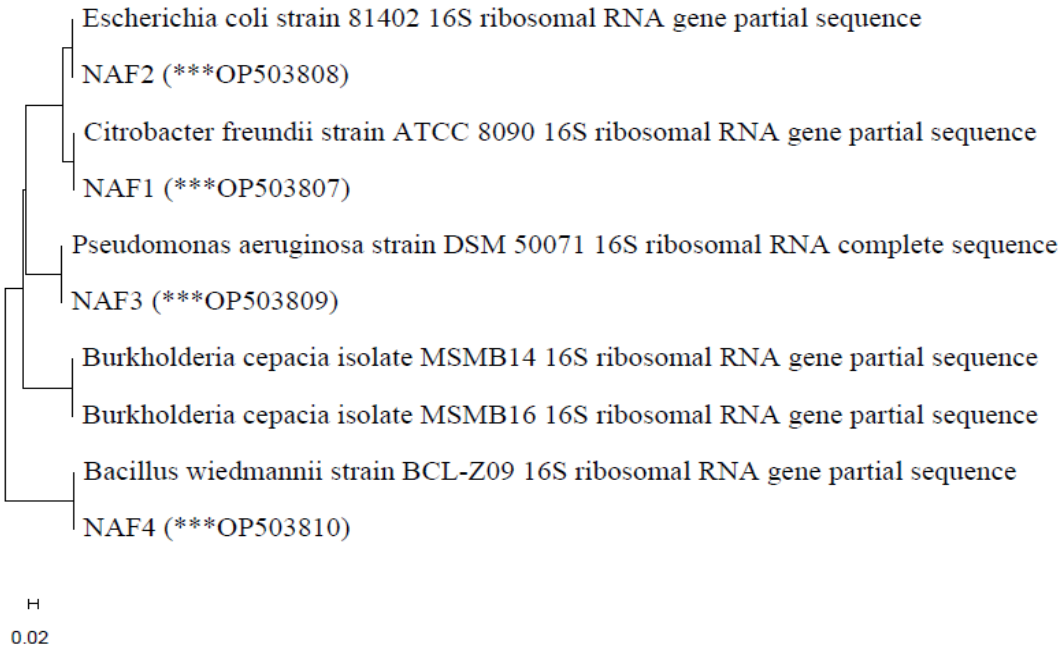


Figure 2. Phylogenetic tree constructed by Neighbor-Joining method derived from analysis of the 16S rRNA gene sequences of dye-degrading bacteria and related sequences obtained from NCBI.

The adequacy of the proposed model was evaluated by the Design expert 11.1.2.0 as depicted in Table 3. The model showed that the quadratic model possesses an *F*-value (53.30) and low p-values (<0.0001) indicating that the model is significant. The model also possesses a high correlation coefficient (R^2) value (0.9813). The adjusted (R^2) value of 0.9632 compares adequately with the predicted R^2 (0.8939) with a moderate agreement since their difference is less than 0.2. The model significance is true if its p-value < 0.05. The adequate precision value of 23.2533 is appreciably high; a value > 4.0 is desirable and reveals a signal toward the adequacy of the model. The linear terms A, B, C, D, AB, AC, CD, A^2, B^2, C^2, and D^2 are the significant terms for the degradation of RBD-5 by *B. wiedmannii* NAF4 as shown in Table 3.

Table 3. Significance of Experimental Parameters on the Degradation of RB5

Source	Sum of Squares	Degree of Freedom	Mean Square	F-value	p-value	
Model	19351.61	14	1382.26	53.30	< 0.0001	significant
A-Temp.	1260.75	1	1260.75	48.61	< 0.0001	significant
B-pH	4840.08	1	4840.08	186.63	< 0.0001	significant
C-Agitation	533.33	1	533.33	20.56	0.0005	significant
D-Inoculum size	161.33	1	161.33	6.22	0.0258	significant
AB	225.00	1	225.00	8.68	0.0106	significant
AC	306.25	1	306.25	11.81	0.0040	significant
AD	100.00	1	100.00	3.86	0.0697	
BC	0.2500	1	0.2500	0.0096	0.9232	
BD	0.0000	1	0.0000	0.0000	1.0000	
CD	441.00	1	441.00	17.00	0.0010	significant
A^2	3906.77	1	3906.77	150.64	< 0.0001	significant
B^2	9264.07	1	9264.07	357.21	< 0.0001	significant
C^2	1443.29	1	1443.29	55.65	< 0.0001	significant
D^2	921.13	1	921.13	35.52	< 0.0001	significant
Residual	363.08	14	25.93			
Residual	363.08	14	25.93			
Pure Error	0.0000	4	0.0000			
Cor Total	19714.69	28				

Mean=50.10; C.V.%=10.16; R^2=0.9813; Adjusted R^2=0.9632; Predicted R^2=0.8939; Adequate Precision=23.2533

The actual response versus predicted response plot for the quadratic model is presented in Figure 3. The data points on the plots were well dispersed along a straight line, which is an indication of strong conformity between the actual and predicted values of the response. This result indicates that the quadratic model is sufficiently adequate in predicting the response variables for the experimental data.

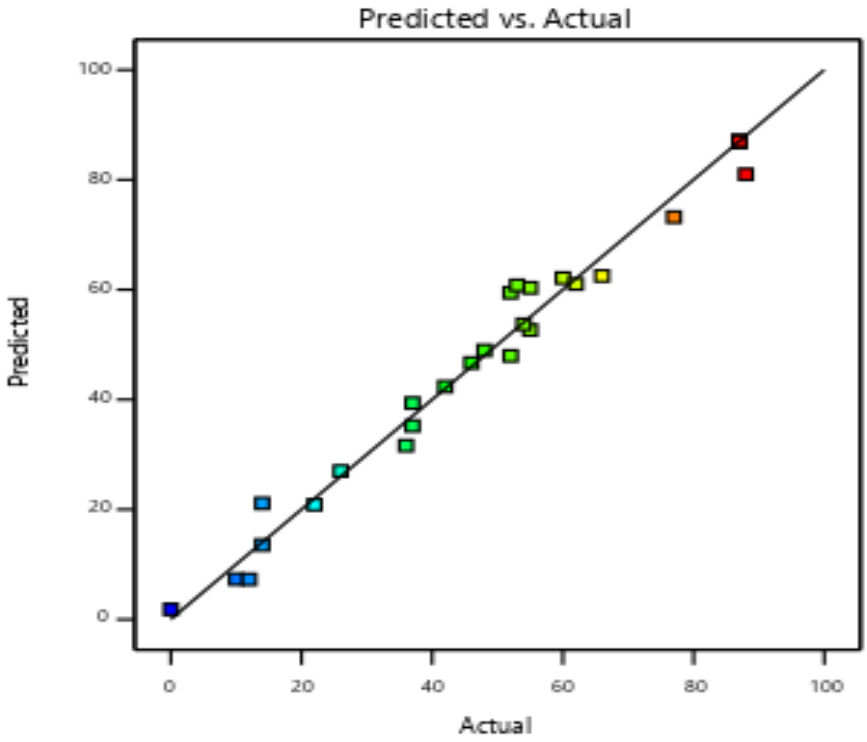


Figure 3. The actual response versus predicted response plot for the quadratic model

The size of the inoculum significantly influences the metabolic activity and microbial composition, serving as a critical determinant in identifying operational factors during the biodegradation process. In this study, the inoculum size ranged between 5 and 15. The percentage of degradation exhibits an increasing trend with an increasing inoculum size, reaching an optimal value of 12.25. However, beyond this optimum threshold, the degradation percentage declines. This decline indicates the presence of overcrowding among the *B. wiedmannii* NAF4 at sizes exceeding the optimal value, resulting in limited interaction with the RB-5 molecules and reduced efficiency of degradation.

Temperature is one of the most important factors affecting the degradation of hazardous chemicals. Bacterial activity and growth for efficient degradation were observed within a temperature range of 30 to 35°C. The optimal degradation temperature for RB-5 in this study was determined to be 29.744°C. Temperature values higher or lower than this optimum significantly impede the viability of BW cells, as depicted in the 3D response surface plots.

The agitation speed influences the degradation of RB-5, increasing it until reaching an equilibrium speed, beyond which degradation decreases. This phenomenon can be attributed to enhanced contact between *B. wiedmannii* and RB-5, facilitating the degradation process. However, further increases in agitation speed beyond the equilibrium point result in decreased degradation due to high speeds negatively affecting cell viability necessary for effective degradation. The response surface plots (Figure 4A-F) were generated to investigate the interactions among operating parameters and their impact on the degradation efficiency of RB-5 by *B. wiedmannii*. The pH of the solution significantly influences the degradation process. There is a gradual increase in the percentage of RB-5 degraded as the pH increases, reaching an equilibrium at pH 7. After pH 7, the degrading ability of *B. wiedmannii* NAF4 decreases, indicating a pH-dependent relationship between *B. wiedmannii* strain NAF4 and the nature of RB-5 in solution (Figure 4A).

The percentage of RBD-5 degraded was observed to increase with an increase in the initial inoculum size at a fixed agitation speed (115 rpm), pH (7), and temperature (30°C). The interactions among all parameters presented in Fig. 4 (A-F) demonstrate a synergistic

relationship. When one parameter increases along with another, the degradation ability of *B. wiedmannii* also increases, suggesting that each parameter plays a significant role in the degradation process.

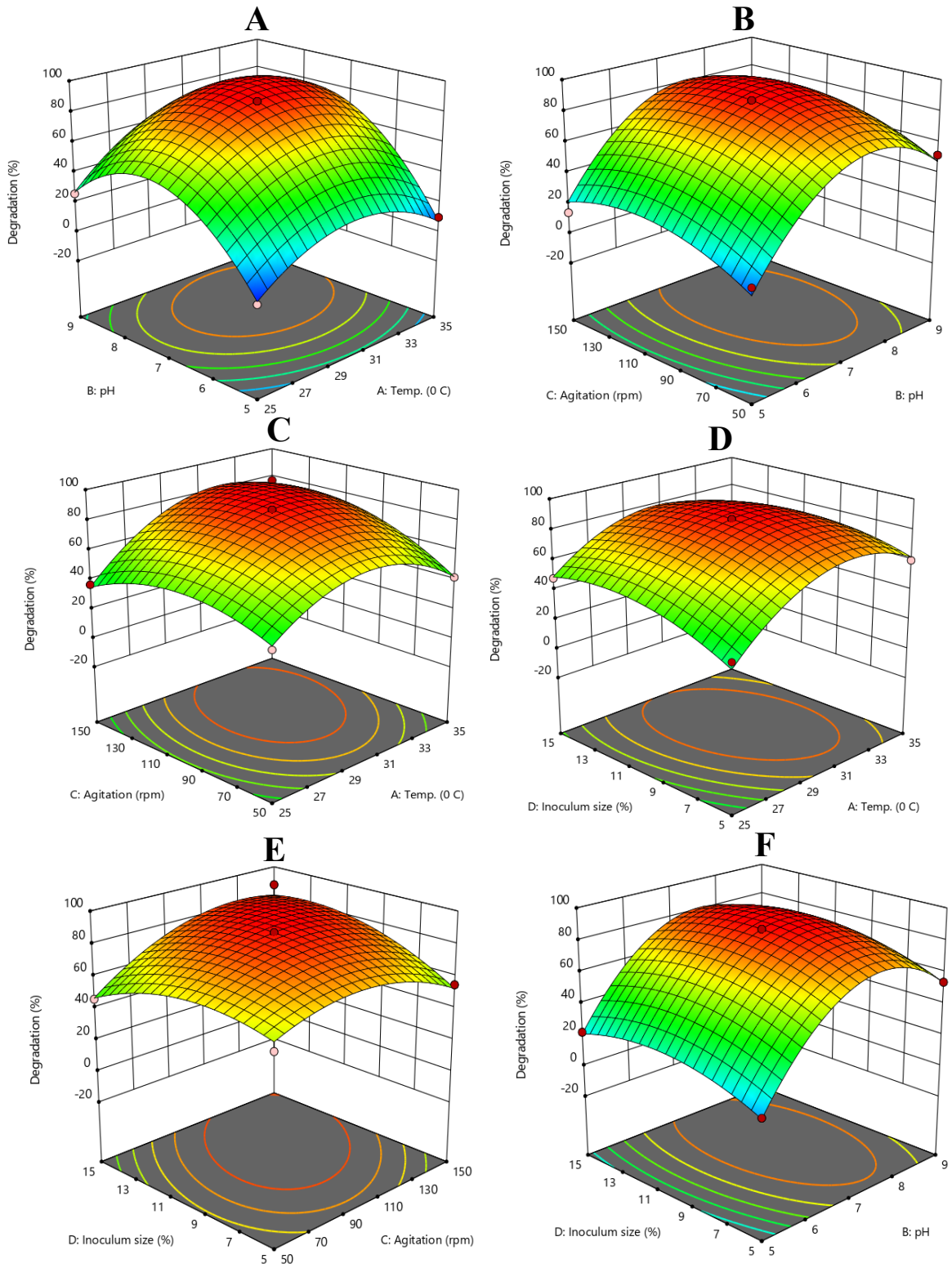


Figure 4. The 3D plots and Contour plots showing the effect of (A). pH and Temperature (B). Agitation and pH (C). Agitation and Temp. (D). Inoculum size and Temp. (E). Inoculum size and Agitation-speed (F). Inocum size and pH

However, there are optimal conditions reached during the degradation process, beyond which the degradation ability of *B. wiedmannii* NAF4 decreases. The optimum conditions identified in this study were an agitation speed of 115.777 rpm, pH of 7.449, inoculum size of 12.255, and temperature of 29.74°C, as illustrated in the Figure 5.

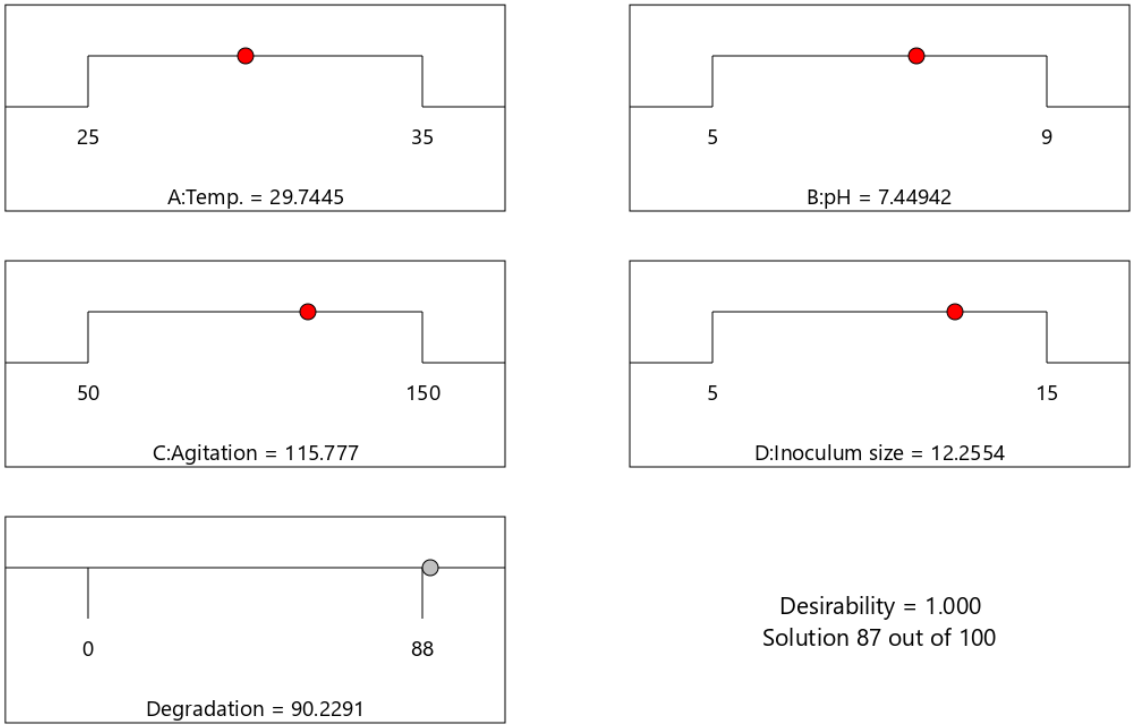


Figure 5: Optimum temp, pH, Agitation and inoculums size required to achieve 90.2291 % of the RB-5 degradation by *Bacillus wiedmannii strain NAF4*

Discussion

Reactive Black 5 (RB5) dye is a commonly used synthetic dye in various industries, including textile, paper, and leather. However, its wide-scale application has raised concerns due to its persistence and adverse environmental effects (Al Sharabati et al. 2021, Marković et al. 2023) Biodegradation, a promising approach to mitigate the environmental impact of RB5 dye, is gaining attention (Li et al. 2021) indicates their potential adaptation to the unique environmental conditions created by textile industry effluents.

The four bacterial isolates, isolated from the soil impacted by wastewater from local textiles through enrichment techniques. Enrichment techniques have been shown to be crucial in enhancing the likelihood of isolating microorganisms capable of degrading pollutants (Bokade et al. 2023). These techniques apply a selective pressure that promotes the enrichment of the desired microbial population possessing the inherent ability to utilize the pollutant as a carbon or energy source. Consequently, this selective pressure encourages the growth of pollutant-degrading microorganisms while simultaneously suppressing the growth of non-degrading or slower-degrading species (Bokade et al. 2023).

These isolates have demonstrated their ability to thrive in an environment enriched with various organic and inorganic compounds present in the wastewater. This also suggests that they possess specific physiological and metabolic characteristics that enable them to withstand the toxic effects of the textile waste contaminants. These bacteria may have developed adaptive

mechanisms to utilize the complex mixture of organic dyes, chemicals, and other pollutants present in the wastewater as a source of carbon and energy ((Giovanella et al. 2020, Bala et al. 2022).

Considering this knowledge, it is plausible that the dominant isolates identified as *C. freundi* NAF1, *E. coli* strain NAF2, *P. aeruginosa* strain NAF3, and *B. wiedmannii* NAF4 may possess similar enzymatic capabilities, facilitating the degradation and removal of textile dyes present in the contaminated soil (Saravanan et al. 2021)

These findings highlight the varying capabilities of the isolates in degrading RB-5 dye, with *B. wiedmannii* strain NAF4 showing the highest efficacy. The decrease in decolorization percentage over time suggests that the degradation process may have reached a plateau or encountered certain limitations also indicating the dynamic nature of the degradation process.

However, all the tested isolates demonstrated the capability to metabolize RB-5, with *B. wiedmannii* strain NAF4 exhibiting the highest degradation potential among them, even surpassing the degradation ability of the consortium. This finding challenges the conventional understanding that co-cultures are generally more efficient in degradation compared to pure cultures Sauer & Marx (2023). The exceptional performance of isolate *B. wiedmannii* strain NAF4 in degrading RB-5 highlights its novelty and suggests its potential as a promising candidate for further study. This study represents the first-ever investigation into the use of *B. wiedmannii* for the degradation of Reactive black dye (RB-5).

Interestingly, the consortium of isolates displayed a slightly lower performance in RB-5 degradation. This could be attributed to the antagonistic effect observed within the consortium. Previous studies have indicated that isolate *B. wiedmannii* strain NAF4 possesses cytotoxic properties, which may have contributed to the observed antagonistic effect within the consortium. This finding suggests that the interplay between different isolates within a consortium can significantly impact the overall degradation efficiency.

Understanding the varying abilities of different isolates in degrading RB-5 dye is important for the development of efficient bioremediation strategies. By identifying isolates with higher decolorization potential, such as *B. wiedmannii* strain NAF4 and *P. aeruginosa* strain NAF3, researchers can further investigate and optimize their performance for industrial applications.

Several studies have employed Response Surface Methodology (RSM) as an optimization tool in the biodegradation of various azo dyes, highlighting its effectiveness in enhancing degradation processes (Kumar et al. 2019, Khatoon and Rai 2020) RSM offers several advantages in the context of biodegradation studies. Firstly, it allows for the simultaneous investigation of multiple variables and their interactions, enabling a comprehensive analysis of the degradation process. This comprehensive approach ensures that all relevant factors are considered, leading to more accurate and reliable results.

To optimize the degradation process of RB-5, the study utilized Response Surface Methodology (RSM) in conjunction with the isolated bacterium strain. RSM is a statistical tool that enables the verification and optimization of process parameters by evaluating their effects on the degradation efficiency. In this case, the process parameters investigated were pH, temperature, inoculum size, and agitation speed (Manogaran et al. 2021).

To visualize the effects of these process parameters, 3D plots and contour plots were generated. The plots depicted the interactions between different pairs of parameters and their influence on the degradation efficiency of RB-5 by *B. wiedmannii*. Specifically, the plots illustrated the effects of pH and temperature, agitation speed and pH, agitation speed and temperature, inoculum size and temperature, inoculum size and agitation speed, and inoculum size and pH.

In this study, the application of RSM for the optimization of process parameters in the degradation of Reactive Black 5 (RB-5) dye by *Bacillus wiedmannii* strain NAF4 is affirmed. By utilizing RSM, this study showed the optimal conditions for maximum dye degradation efficiency. The identified optimal conditions included an agitation speed of 115.777 rpm, pH

of 7.449, inoculum size of 12.255, and temperature of 29.74°C. These conditions were shown to significantly enhance the degradation of RB-5, resulting in a maximum degradation efficiency of 90.2291%.

The generated response surface plots not only allow for the determination of optimum conditions but also provide valuable information regarding the interactions between the different operating parameters. The plots reveal the synergistic relationships among pH, temperature, inoculum size, and agitation speed, emphasizing the intricate interplay of these factors in the degradation process.

The identified optimum conditions hold great significance for practical applications. They serve as guidelines for optimizing RB-5 degradation processes using *B. wiedmannii* in real-world scenarios. Implementing these conditions can lead to enhanced degradation efficiency and more sustainable approaches for the remediation of RB-5-contaminated environments. Overall, this study demonstrates the immense potential of *B. wiedmannii i*n degrading RB-5 dye. The organism's enzymatic capabilities, as evidenced by the production of laccase, lignin peroxidase, manganese peroxidase, and azoreductase, indicate its suitability as a biodegradation agent. The utilization of RSM and response surface plots further enhances our understanding of the interactions between process parameters and their effects on RB-5 degradation. The identified optimum conditions provide practical insights for optimizing the degradation process and highlight the promising applications of *B. wiedmannii* in addressing RB-5 contamination challenges. Furthermore, the utilization of RSM in this study serves as an innovative approach to maximize the degradation process by optimizing degradation parameters. The successful application of RSM in this study demonstrates its effectiveness as an optimization tool and provides valuable insights for future biodegradation studies. The optimized conditions identified through RSM offer practical guidance for the implementation of efficient and environmentally friendly strategies for the degradation of RB-5 and potentially other similar azo dyes.

Conclusions

The Biodegradation study of RB-5 was carried out with the bacterium *B. wiedmannii* NAF4 strain isolated from the dye- contaminated field soil. The RSM was used with isolated bacterium strain for the verification, and optimization, of process parameters, namely pH, temperature, Inoculum size and agitation-speed for degradation of RB-5. This statistical analysis technique confirmed as useful and powerful tool, in standardizing optimum degradation conditions. This is the first research which describes the application of statistical designing tool for the degradation of RB-5 by *B. wiedmannii* NAF4 strain culture and Design expert software (new version 10.0.1). The optimum conditions identified in this study were an agitation speed of 115.777 rpm, pH of 7.449, inoculum size of 12.255, and temperature of 29.74°C. From the results, it can be concluded that RSM applied here as optimization tool can be effectively used elsewhere as well, to maximize the degradation process by optimizing degradation parameters.

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Competing interests: The authors declare that they have no known competing financial interests or personal relationships which have, or could be perceived to have, influenced the work reported in this article.

Funding: No funding was received

Authors' contributions: **AAT:** Conceived and designed the experiments, Manuscript preparation. **MN and AWT:** Data analysis and interpretation, Manuscript preparation. **JAA:**

Contributed reagents/materials/analysis tools; All authors carried out laboratory experiment. All authors read and approved the final manuscript.

Acknowledgements: The authors express profound gratitude to their Institution management for creating an enabling environment

References

Ahsan Z., Kalsoom U., Bhatti H.N., Aftab K., Khalid N., Bilal M. 2021. Enzyme-assisted bioremediation approach for synthetic dyes and polycyclic aromatic hydrocarbons degradation. Journal of Basic Microbiology. 61(11): 960-981.

Al-Tohamy R., Ali S.S., Li F., Okasha K.M., Mahmoud Y.A.G., Elsamahy T., ..., Sun J. 2022. A critical review on the treatment of dye-containing wastewater: Ecotoxicological and health concerns of textile dyes and possible remediation approaches for environmental safety. Ecotoxicology and Environmental Safety. 231: 113160.

Bhutia M.O., Thapa N., Tamang J.P. 2021. Molecular characterization of bacteria, detection of enterotoxin genes, and screening of antibiotic susceptibility patterns in traditionally processed meat products of Sikkim, India. Frontiers in Microbiology. 11: 599606. https://doi.org/10.3389/fmicb.2020.599606

Bokade P., Gaur V.K., Tripathi V., Bobate S., Manickam N., Bajaj A. 2023. Bacterial remediation of pesticide polluted soils: Exploring the feasibility of site restoration. Journal of Hazardous Materials. 441: 129906.

Dixit P., Lal R.C. 2019. A critical analysis of Indian textile industry: An insight into inclusive growth and social responsibility. Russian Journal of Agricultural and Socio-Economic Sciences. 88(4): 53-61.

Elgarahy A.M., Elwakeel K.Z., Mohammad S.H., Elshoubaky G.A. 2021. A critical review of biosorption of dyes, heavy metals and metalloids from wastewater as an efficient and green process. Cleaner Engineering and Technology. 4: 100209.

Elumalai P., Parthipan P., Huang M., Muthukumar B., Cheng L., Govarthanan M., Rajasekar A. 2021. Enhanced biodegradation of hydrophobic organic pollutants by the bacterial consortium: Impact of enzymes and biosurfactants. Environmental Pollution. 289: 117956.

Espinosa-Ortiz E.J., Rene E.R., Gerlach R. 2022. Potential use of fungal-bacterial co-cultures for the removal of organic pollutants. Critical reviews in biotechnology. 42(3): 361-383.

Franco-Duarte R., Černáková L., Kadam S., Kaushik K., Salehi B., Bevilacqua A., ..., Rodrigues C.F. 2019. Advances in chemical and biological methods to identify microorganisms—from past to present. Microorganisms. 7(5):130.

Gaur N., Narasimhulu K., PydiSetty Y. 2018. Recent advances in the bio-remediation of persistent organic pollutants and its effect on environment. Journal of cleaner production. 198: 1602-1631.

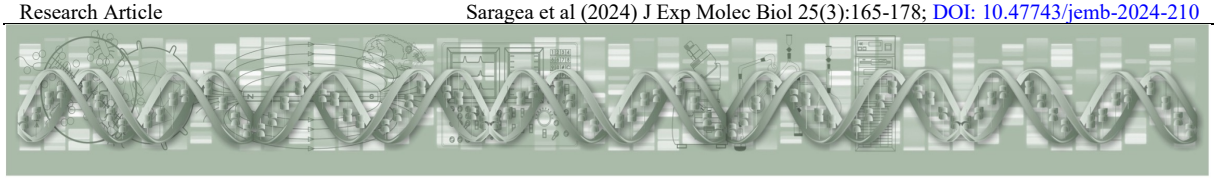
Khan M.D., Singh A., Khan M.Z., Tabraiz S., Sheikh J. 2023. Current perspectives, recent advancements, and efficiencies of various dye-containing wastewater treatment technologies. Journal of Water Process Engineering. 53: 103579.

Khan S., Malik A. 2018, Toxicity evaluation of textile effluents and role of native soil bacterium in biodegradation of a textile dye. Environmental Science and Pollution Research. 25: 4446-4458.

Khatoon H., Rai J.P.N. 2020. Optimization studies on biodegradation of atrazine by *Bacillus badius* ABP6 strain using response surface methodology. Biotechnology reports. 26: e00459.

Kumar N., Sinha S., Mehrotra T., Singh R., Tandon S., Thakur I.S. 2019. Biodecolorization of azo dye Acid Black 24 by *Bacillus pseudomycoides*: Process optimization using Box Behnken design model and toxicity assessment. Bioresource Technology Reports. 8: 100311.

Manogaran M., Yasid N.A., Othman A.R., Gunasekaran B., Halmi M.I.E., Shukor M.Y.A. 2021. Biodecolourisation of Reactive Red 120 as a sole carbon source by a bacterial consortium—Toxicity assessment and statistical optimisation. International journal of environmental research and public health. 18(5): 2424.
Moosvi S., Keharia H., Madamwar D. 2005. Decolourization of textile dye Reactive Violet 5 by a newly isolated bacterial consortium RVM 11.1. World Journal of Microbiology and Biotechnology. 21: 667-672.
Pérez García P.M., Ibáñez-Calero S.L., Escalera Vásquez R. 2017. Degradation of synthetic organic dyes in solution by ferrate-hypochlorite or calcium hypochlorite. Investigación & Desarrollo. 1(17): 43-53.
Sheela T., Sadasivam S.K. 2020. Dye degradation potential and its degradative enzymes synthesis of *Bacillus cereus* SKB12 isolated from a textile industrial effluent. Journal of Applied Biology and Biotechnology. 8(3): 42-46.
Srivastava A., Bandhu S. (2022). Biotechnological advancements and challenges in textile effluents management for a sustainable bioeconomy: Indian case studies. Case Studies in Chemical and Environmental Engineering: 100186.
Yusuf M. 2019. Synthetic dyes: a threat to the environment and water ecosystem. Textiles and clothing: 11-26.
Zhao H.P., Wu Q.S., Wang L., Zhao X.T., Gao H.W. 2009. Degradation of phenanthrene by bacterial strain isolated from soil in oil refinery fields in Shanghai China. Journal of hazardous materials. 164(2-3): 863-869.



ALZHEIMER'S DISEASE AS A MULTIFACTORIAL NEURODEGENERATIVE PATHOLOGY - A COHORT STUDY

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Abstract

Situated within the broad spectrum of neurodegenerative disorders, Alzheimer's disease (AD) is known for its complexity, heterogeneity, multiple genetic mutations, epigenetic and biochemical modifications, and irreversible progression from early stages characterized by deficits in the ability to encode and store new information to subsequent progressive cognitive, functional, and behavioral decline. The conducted study aimed to compile conclusive statistics, identify genetic factors, and correlate them with environmental ones, thus highlighting the importance of developing evaluation programs and early introduction of medication to decelerate the progression of neurodegenerative processes. The analytical, observational, retrospective study was conducted on an extended cohort of 2277 patients admitted with chronic neurological diseases to the Neurology Department of "Dr. Iacob Czihac" Clinical Military Emergency Hospital Iași from January 1, 2022, to December 31, 2023. Among these, 219 patients were diagnosed with AD at various stages. Forty-three cases exhibiting genetic predisposition (19.63%) were selected and thoroughly analyzed based on medical records. The study emphasizes the significant position of AD among chronic neurological diseases. Although the majority do not present hereditary antecedents (80.36%), predisposing conditions, environmental factors, stress, and the region of residence play fundamental roles in the disease's determinism. It is observed that individuals in the 60-70 age category (71.23%) from urban areas (63.01%), especially females (63.47%), have a higher probability of developing AD. Maternally transmitted AD prevalence was 58.13%, while paternally inherited AD accounted for 32.55%, with only 4 cases having antecedents on both lines (9.30%). Unequivocally characterized by a vast etiology, AD is a multifactorial disorder resulting from the bilateral interaction and continuous corroboration of genetic and environmental factors.

Keywords: Alzheimer, Neurodegeneration, Multifactorial, Dementia, Hereditary

Introduction

Initially described in 1906 by the pathologist and psychiatrist Alois Alzheimer as a "disease of forgetting," the eponymous condition is first recognized by short-term memory impairment, followed by a progressive decline in cognitive, functional, and behavioral abilities. The disease involves deterioration in reasoning, memory, and visual perception, along with difficulties in focusing attention and maintaining social interactions. As the disease advances to moderate or severe stages, patients may experience symptoms such as agitation, delusional thoughts, anxiety, depression, irritability, apathy, hallucinations, delirium, and insomnia (Maurer et al. 1997, Burns et al. 2002, Hippius and Neundörfer 2003, Szalontay et al. 2005, Soria Lopez et al. 2019, Szalontay 2014).

Received 28 August 2024; Revised 5 September 2024; Accepted 6 September 2024;
Published Online 10 September 2024; Published 9 October 2024

Situated within the broad spectrum of neurodegenerative disorders, AD is recognized for its heterogeneity, underlying hypotheses, numerous genetic mutations, epigenetic and biochemical alterations, and irreversible progression from early stages characterized by deficits in the ability to encode and store new information to subsequent progressive cognitive, functional, and behavioral decline in advanced, terminal stages. It is unequivocally characterized by a vast etiology, including cerebral atrophy associated with neurotrophin depletion, mitochondrial dysfunction, and the accumulation of senile plaques and intraneuronal neurofibrillary tangles as a result of autosomal dominant mutations (APP, PSEN1, PSEN2) or the presence of allelic variants (APOE4, SORT1, MAPT, APOJ).

As one of the major causative factors of progressive, irreversible dementia, the pathogenesis of AD is predominantly attributed to the presence of intracellular neurofibrillary tangles, composed of hyperphosphorylated tau protein, and dense or diffuse extracellular β-amyloid ("senile") plaques within the limbic and cortical areas. AD is classified as a neurodegenerative disorder primarily driven by protein misfolding, where conformational changes are induced by a combination of genetic and non-genetic factors, including physiological, biological, demographic, behavioral, pathological, and environmental influences. The principal mechanism underlying the disease is the hyperphosphorylation, aberrant polymerization, and processing of normal soluble proteins (Tiwari et al. 2019).

AD is characterized by a complex and extensive etiology, attributable to both genetic and environmental factors. Genetic mutations on chromosomes 14, 1, 19, and 21 play significant roles, with autosomal-dominant inheritance patterns being common—AD often follows a monogenic inheritance model for chromosomes 21, 14, and 1. In addition to genetic predispositions, the disease may be induced by exposure to toxic substances (such as aluminum, pesticides, and organic compounds), infectious agents (including herpes virus and lentiviruses), and cranio-cerebral trauma. The development of amyloid plaques, cerebrovascular amyloidosis, and cerebrovascular accidents, particularly in mixed forms, also contributes to the pathogenesis of AD. Moreover, several pathological mechanisms are implicated in the progression of AD, including acute-phase inflammatory responses, the formation of amyloid plaques and neurofibrillary tangles of protein tau, and significant deficits in choline acetyltransferase and acetylcholine, leading to marked neurochemical and cholinergic deficiencies. Structural and cytoskeletal impairments in mitochondria—key organelles responsible for oxidative phosphorylation and energy release—resulting from neurofibrillary accumulations, as well as metabolic lesions, the proliferation of apolipoprotein E4, and imbalances in neurotransmitters such as norepinephrine, dopamine, and serotonin, can all initiate and exacerbate the neurodegenerative processes associated with AD (Szalontay et al. 2005, Szalontay 2014, Masters et al. 2015).

This study is driven by the pressing need to deepen our understanding of the underlying mechanisms of AD, a neurodegenerative disorder with a profound impact on global public health. Current treatment options remain limited and are not curative, highlighting the urgent need for novel therapeutic approaches. The increasing prevalence of AD, along with its significant social and economic consequences underscores the importance of advancing research in this area.

The purpose of the retrospective study was to establish conclusive statistics and to identify/observe both genetic and non-genetic factors. It encompasses individuals admitted to the Neurology Department of the "Dr. Iacob Czihac" Clinical Military Emergency Hospital in Iași over a two-year period (from January 1, 2022, to December 31, 2023).

With the continuous global aging of the population (corresponding to an increase in life expectancy), scientific research has increasingly focused on identifying the factors that contribute to the onset of AD, a neurodegenerative disease, as well as elucidating its underlying mechanisms and exploring potential treatment options. The rising incidence and mortality rates

further underscore the importance of these investigative efforts (Li et al. 2022, Gustavsson et al. 2023).

Although there are no effective methods to halt the progression of AD, which remains irreversible and progressive with variable rates of decline, published studies demonstrate that up to one-third of dementia cases could potentially be prevented or the onset delayed by understanding controllable risk factors. This involves meticulous population monitoring and screening, the identification of genetic and non-genetic determinants, and the systematic, comprehensive reporting of epidemiological trends, all of which are essential components in addressing this intricate, multifaceted, and evolving process (Gauthier et al. 2016, GBD 2019 Dementia Forecasting Collaborators 2019, GBD 2019 Diseases and Injuries Collaborators 2020, GBD 2019 Risk Factors Collaborators 2020, Livingston et al. 2020, Gao and Liu 2021, Li et al. 2022, Gustavsson et al. 2023).

Materials and Methods

Characterization of the study cohorts

The retrospective study includes individuals who were admitted to the Neurology Department of the "Dr. Iacob Czihac" Clinical Military Emergency Hospital in Iași between January 1, 2022, and December 31, 2023.

Patients were selected according to their diagnoses, specifically targeting cases of chronic neurological disorders, with a particular emphasis on AD, from the hospital's databases. The selection focused on individuals who exhibited a familial genetic predisposition to neurodegenerative diseases.

During the years 2022 and 2023, a total of 2277 individuals with chronic neurological conditions were admitted, 219 of whom were diagnosed with AD. For the purpose of the genetic study, 43 cases exhibiting genetic predisposition were selected, and their medical records were analyzed: 25 cases with maternal inheritance, 14 cases with paternal inheritance, and 4 cases with a history of neurodegenerative conditions (AD) in both maternal and paternal lines.

In 2022, out of the 121 patients admitted with AD, only 22 individuals were identified with varying degrees of genetic predisposition, distributed as follows: 13 cases with maternal inheritance, 7 cases with paternal inheritance, and 2 cases where both parents had previously been diagnosed with AD. In 2023, in Iași, of the 98 patients with AD, only 21 had genetic predisposition, including 12 cases with antecedents on the mother’s side, 7 cases with antecedents on the father’s side, and 2 cases where the genetic predisposition or history was traced through both the maternal and paternal lineages.

Thus, the study cohort for the year 2022 included 1265 patients with various forms of chronic neurological disorders, whereas in 2023, a smaller number of 1012 individuals were admitted. Among the 1265 patients hospitalized in 2022, a final diagnosis of AD was established in 121 cases at various stages. Over the subsequent 12 months (i.e., in 2023), 98 individuals with AD were identified out of a total of 1012 patients admitted (at their first hospitalization). The prevalence of AD diagnosis in 2022 was 9.5652%, a trend that continued into the following year, with a prevalence of 9.6837%. The study thus highlights that AD holds a significant position among chronic neurological disorders, with 9.6179% of patients admitted with chronic neurological conditions being diagnosed with early-onset, late-onset, atypical, mixed, or unspecified AD over the two-year period.

Methods applied to conduct the research

The methodology comprised a comprehensive analysis of medical records, including the results of supplementary investigations, cognitive assessments, and general clinical examinations, as well as neurological, psychiatric, and neuropsychological evaluations. This process also

involved the extraction of relevant, pertinent information. The medical data recorded in the patients' electronic files were processed, and the inclusion criteria comprised hospitalized patients who had been diagnosed with AD. The overarching objective of this study was to generate conclusive statistical insights. The relevant data for this retrospective study were graphically illustrated and tabulated to enhance the visualization of the observations derived from the analyzed information.

Results and discussions

The observational, analytical, retrospective study includes individuals admitted to the Neurology Department of the "Dr. Iacob Czihac" Clinical Military Emergency Hospital in Iași between January 1, 2022, and December 31, 2023, as of the date of their last admission (Figure 1).

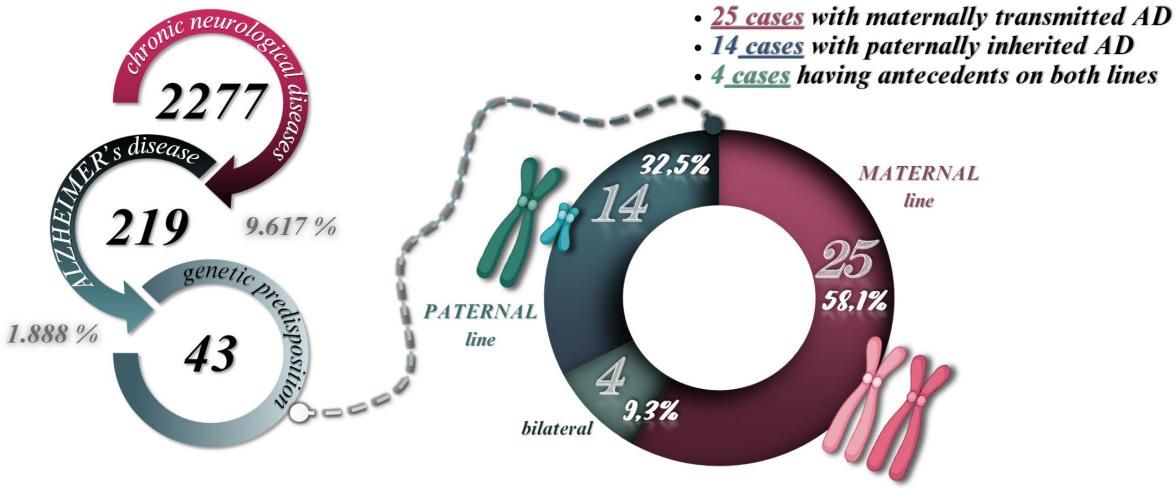


Figure 1. The numerical distribution of the cohort from Iași according to diagnosis and the hereditary lineage involved in the genetic susceptibility transmission process

During the two-year period, a total of 2277 individuals with chronic neurological conditions were admitted. In 2022, 1265 cases were recorded, whereas in 2023, only 1012 cases were documented. This observation indicates a slight decrease in the number of newly diagnosed and hospitalized patients (a difference of 253 cases) between the two years during which the statistical data were collected (Table 1).

Among the total of 2277 patients admitted with chronic neurological conditions (at the onset of their illnesses), 219 were diagnosed with AD (121 patients in 2022 and 98 patients in 2023). In accordance with the inclusion criteria outlined in the preceding section, 43 individuals exhibiting genetic predisposition were selected, and their medical records were thoroughly analyzed. Upon analyzing the medical records and focusing on individuals with familial predisposition, it was determined that the genetic predisposition was inherited as follows: 25 cases through the mother's side of the family, 14 cases through the father's side of the family, and in the remaining 4 cases, both parents were diagnosed with the same neurodegenerative disorder (AD) (Figure 1, Figure 2, Table 1).

Table 1. Patients admitted with chronic neurological conditions-"Dr. Iacob Czihac" Clinical Military Emergency Hospital Iași-2022 and 2023

	Total number of patients with chronic neurological conditions	*Total number of patients with AD*	*Without genetic / familial predisposition*	*With genetic or familial predisposition*	*Familial history of AD on:*		
					mother's side of the family	*father's side of the family*	*both sides of the family*
2022	1265	121	99	22	13	7	2
2023	1012	98	77	21	12	7	2
TOTAL	***2277***	***219***	***176***	***43***	***25***	***14***	***4***
Percentage of the total number of patients (2,277)		9,6179 %	7,7294 %	1,8884 %	1,09 %	0,61 %	0,17 %
Percentage of the total number of patients with AD (219)			80,3652 %	19,6347 %	11,4155%	6,3926 %	1,8264 %
Percentage of the total number of patients with genetic predisposition (43)					58,1395 %	32,5581 %	9,3023 %

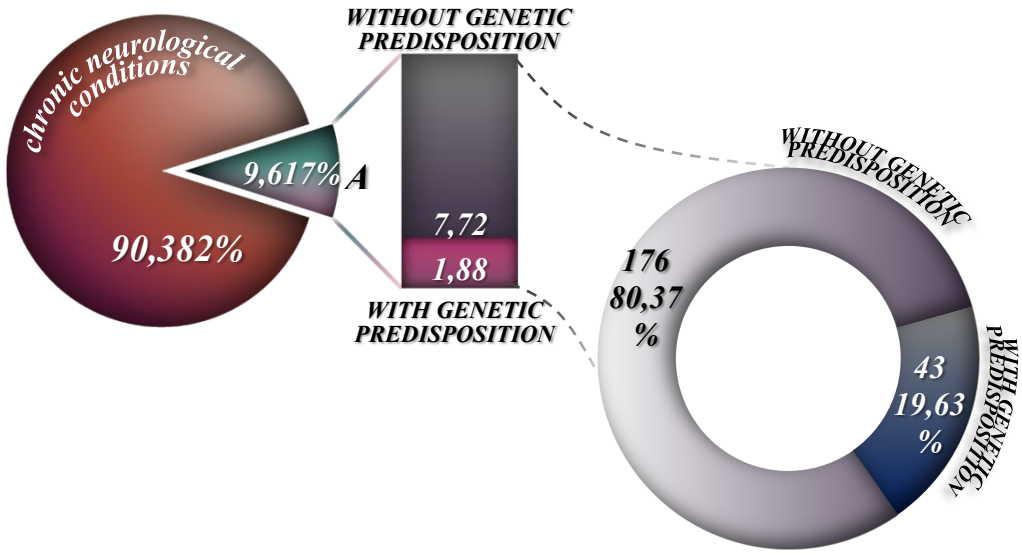


Figure 2. Distribution of AD Patients with or without Genetic Predisposition

Epidemiological Analysis

The study emphasizes the significant position of AD among chronic neurological diseases. Analyzing the data, it is observed that 2058 patients, representing a percentage of 90.382%, were diagnosed with other chronic neurological conditions, including various forms of dementia, whereas only 9.61% were diagnosed with AD (219 patients) (Figure 2). From an etiological perspective, dementias are a heterogeneous group of systemic or neurological conditions affecting the central nervous system, including forms such as AD, mixed dementias (AD associated with cerebrovascular disease or with Lewy bodies), vascular dementias, α-synucleinopathies (Lewy bodies and dementia associated with Parkinson's disease), as well as inflammatory, infectious, metabolic, and neoplastic diseases.

Demographic and Physiological Risk Factors

- **Gender distribution of patients diagnosed with AD**

The analysis of medical records reveals that out of the 219 patients diagnosed with AD at various stages, 139 were female (63.47%), while the remaining 80 individuals were male (36.52%) (Table 2).

Table 2. Gender Distribution Based on the Year of AD Diagnosis

	Total number of patients with chronic neurological conditions	Total number of patients with AD	Women	Men
2022	1265	121	78	43
2023	1012	98	61	37
TOTAL	**2277**	**219**	**139**	**80**

The breakdown of this patient cohort across the two years yields the following observations: in 2022, out of the total 121 patients diagnosed with AD, 78 were women (64.46%) and 43 were men (35.53%). In contrast, in 2023, among the 98 patients with AD, there were 61 women (62.24%) and only 37 men (37.75%) (Table 2).

Consequently, a higher incidence is observed among females (63.47% of the diagnosed AD cases), a trend also supported by globally published statistics. The female sex is considered a physiological risk factor for AD. The disparity in sex distribution in favor of females becomes increasingly pronounced as the incidence rate escalates (Figure 3).

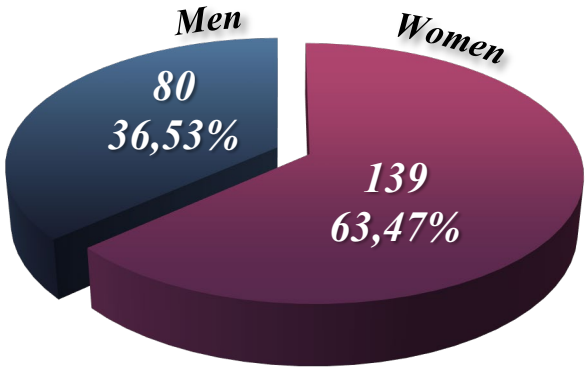


Figure 3. Physiological risk factors: distribution by sex of patients admitted between January 1, 2022, and December 31, 2023

- **Age distribution of patients diagnosed with AD**

The distribution across age groups is fundamentally associated with risk factors involved in the etiology of this progressive condition, characterized by minor or major neurocognitive disturbances (Stănescu, 2015; Szalontay, 2014) (Table 3).

Table 3. The distribution by age of patients diagnosed with AD

AGE	***No. of CASES***	***DIAGNOSIS***
< 60 years	***20***	AD - presenile dementia
60-70 years	***156***	AD
> 70 years	***43***	AD - senile dementia

The analysis of the interdependence between the age of onset or diagnosis and the type of AD reveals that, in most cases, the disease is detected after the manifestation of specific symptoms (including significant impairment of memory, visual perception, language, attention, reasoning, communication, and behavior) during the seventh decade of life (60-70 years: 71.23%) (Figure 4). This statistical finding aligns with the data available in the scientific literature.

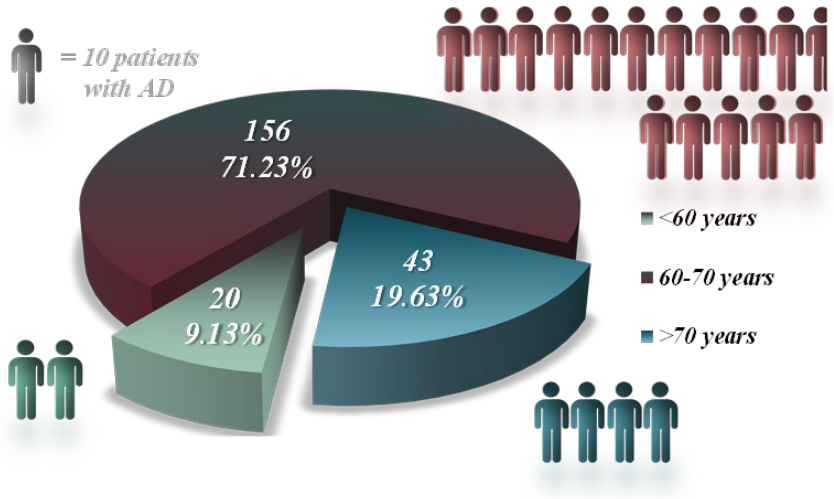


Figure 4. Physiological risk factors: distribution by age groups

Pathological, Behavioral, and Physiological Risk Factors

The analysis of pathological factors was conducted based on their heightened risk of developing AD. Within the high-risk group, 153 individuals with predisposing conditions such as obesity, hypertension, hypercholesterolemia, type 2 diabetes, and previously identified cranio-cerebral trauma, 34 individuals subjected to daily stress, 62 cases of chronic alcoholism, 61 smokers, 29 with aluminium exposure, 38 patients exposed to pesticides, and 16 with exposure to organic solvents were selected (Figure 5).

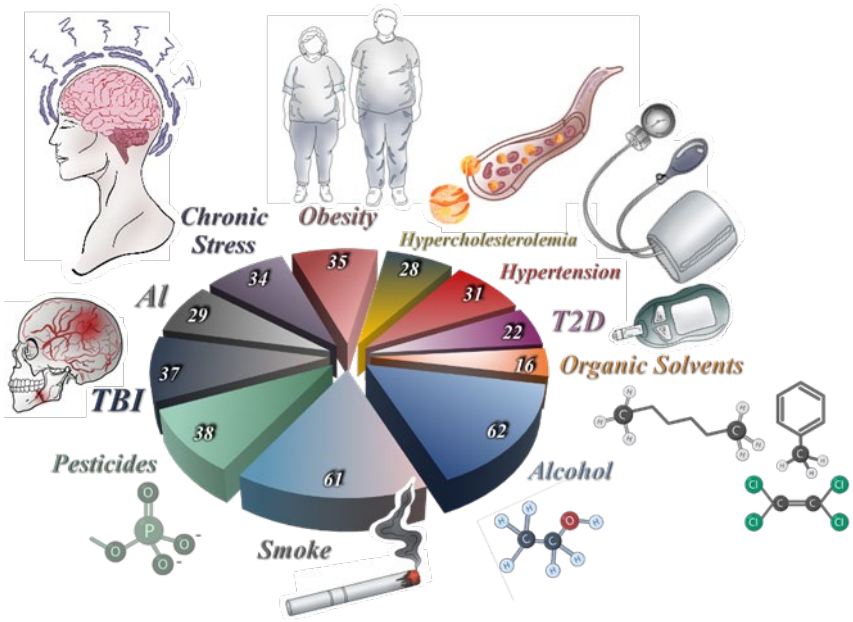


Figure 5. Numerical distribution of individuals exposed to environmental risk factors

Environmental Risk Factors

The study conducted in Iași reveals a notable predominance of AD diagnoses among individuals from urban areas (Table 4), with approximately 63% of the cases, corresponding to 138 patients, originating from these environments. Conversely, only 81 cases, representing 36.98%, were identified in patients from rural areas (Figure 6).

Table 4. Distribution by area and year of admission

	Urban areas	*Rural areas*
2022	69	52
2023	69	29
TOTAL	138	81

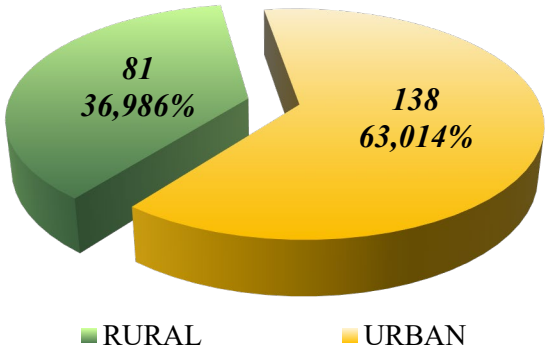


Figure 6. The distribution according to the place of residence of patients diagnosed with AD

In both years, the number of patients admitted from urban environments remained constant (69 patients). In contrast, a decline in the diagnosis of AD among patients from rural areas was noted (Figure 7). The lower number of patients from rural areas, especially in the case of early dementia diagnosis (and the higher prevalence of individuals from urban areas), is attributed in this case to the lower level of medical education and proactive decision-making in rural settings.

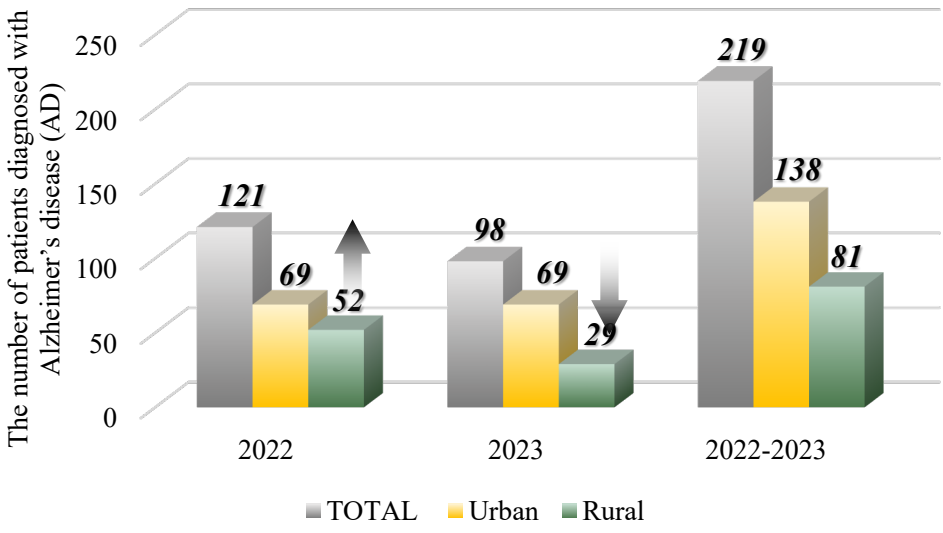


Figure 7. Numerical distribution of patients based on place of residence

Genetic Factors in the Pathogenesis of AD

The analysis of patient cohorts reveals that genetic predisposition contributed to the development of AD in 43 patients (Figure 8): 22 in 2022 and 21 in 2023 (Table 1). However, the risk levels varied depending on the number of relatives previously diagnosed with AD.

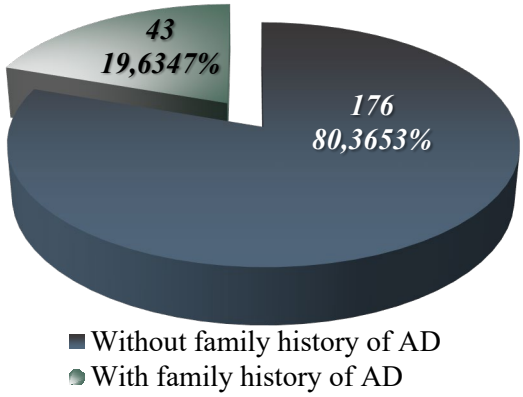


Figure 8. Distribution of Patients Based on Familial Predisposition

Following a detailed examination of the 43 patients with genetic predisposition (Figure 9), it was determined that:

- 4.65% of individuals (2 cases) had only second- or third-degree relatives affected by AD: one patient had a grandparent diagnosed with AD, while the other had an uncle affected by neurodegenerative processes.
- 51.16% of individuals (22 cases) had only one parent previously diagnosed with AD (prior to the genetic consultation and hospitalization).
- 25.58% of individuals (11 cases) had both a parent and a second-, third-, or fourth-degree relative affected by AD: 2 individuals had a fourth-degree relative (a cousin), 2 had a third-degree relative (an aunt or uncle), and 7 had a second-degree relative (a sibling or grandparent).
- The remaining 8 cases had either both parents diagnosed with AD or one parent and at least two second- or third-degree relatives with AD (increased risk).

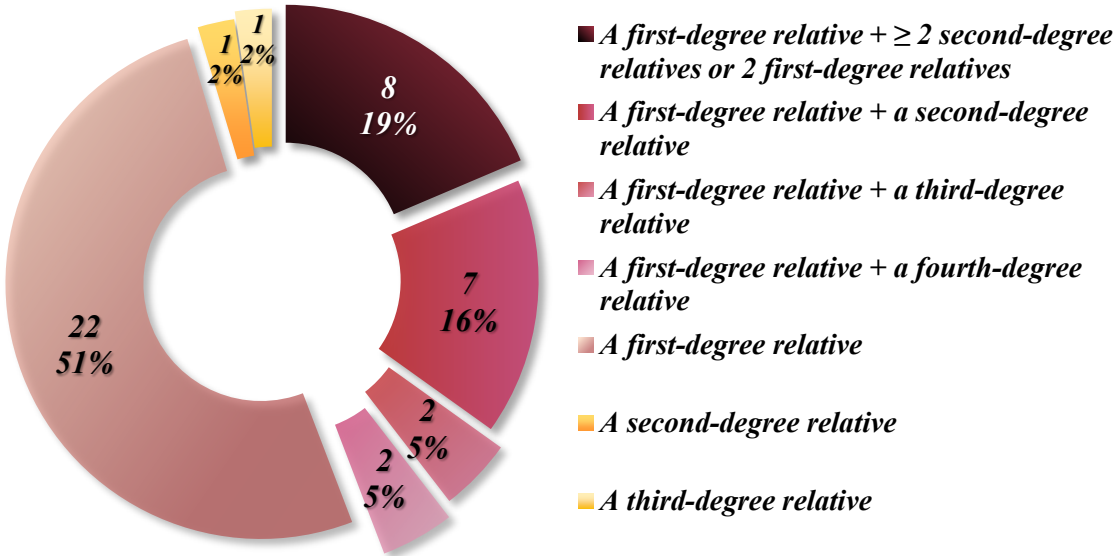


Figure 9. Distribution of patients based on the number of relatives diagnosed with AD

Calculation of Recurrence Risk

In the case of AD, genetic factors are represented by multiple risk genes with minor but additive effects, which collectively contribute to the susceptibility to the disease. The number of these genes is highly variable. Thus, multifactorial determinism involves a complex, continuous interaction between environmental and hereditary components with polygenic influences (the presence of multiple risk genes). In some cases, neurodegenerative processes are triggered in susceptible individuals by exposure to certain environmental factors, leading to the onset of the disease and its specific symptoms.

Reaching and surpassing the risk threshold is dependent on the number of mutant genes and the allelic variants possessed or inherited by an individual. The assessment of genetic risk in AD is calculated similarly to general risks, in accordance with the values specified in the recurrence risk tables for multifactorial diseases (Table 5).

Table 5. Recurrence Risks in Multifactorial Diseases: AD is associated with a frequency of 0.5% and a heritability of 60% (Smith 1972 cited in Covic et al. 2004)

Disease Frequency in Population (%)	***Heritability (%)***	***No. of parents diagnosed with AD***								
		0			***1***			***2***		
		No. of siblings diagnosed with AD								
		0	***1***	***2***	***0***	***1***	***2***	***0***	***1***	***2***
1.0	***80***	0.9	6.7	14.6	8.5	18.7	27.9	40.3	45.9	50.6
	60	1.0	4.9	10.6	5.7	12.3	19.2	21.7	28.3	34.1
	40	1.0	3.3	6.5	3.5	7.0	12	9.7	14.1	18.7
0.5	***80***	0.5	5.1	12.3	6.2	15.5	24.3	37.6	43.2	47.9
	60	***0.5***	***3.4***	***8.4***	***3.8***	***9.5***	***15.8***	***18.1***	***24.4***	***30.0***
	40	0.5	2.1	4.5	2.2	4.9	8.3	7.0	10.8	14.9
0.1	***80***	0.1	2.6	8.4	2.9	9.8	17.6	30.4	36.7	41.2
	60	0.1	1.5	4.6	1.5	5.0	9.6	11.5	17.1	22.2
	40	0.1	0.7	0.7	0.7	2.2	4.2	3.6	6.0	8.7

An important objective of the study was to calculate the recurrence risk (a procedure conducted in accordance with the information provided by empirical risk tables for multifactorial diseases - population frequency of approximately 0.5% and a heritability of 60%), which was estimated to be empirical, between 2-4% (Table 6) for the majority of the studied cases without affected relatives (out of the total 219 patients admitted and diagnosed with AD in 2022 and 2023, 176 had no family history of AD).

Table 6. Risk of recurrence for the studied cases

No. of relatives diagnosed with AD	Year	No. of patients	Recurrence risk	Risk level	Genetic cause
Without family history of AD	2023	77	***2%***	empirical	mutagenesis
	2022	99			
	TOTAL	***176***			
A third-degree relative (uncle)	2022	***1***	***2-3.4%***	very low	genealogical / genetic transmission
A second-degree relative (grandparent)	2022	***1***	***3.4%***	low	

A first-degree relative (parent)	2023	10	***3.8%***	minimal / slight
	2022	12		
	TOTAL	***22***		
A first-degree relative (mother/father) + a fourth-degree relative (cousin)	2022	***2***	***3.8-9.5%***	moderate
A first-degree relative (mother/father) and a third-degree relative (aunt/uncle)	2022	***2***	***3.8-9.5%***	
A first-degree relative (parent) and a second-degree relative (sibling/grandparent)	2023	5	***9.5%***	
	2022	2		
	TOTAL	***7***		
A first-degree relative (parent) and at least two second-degree or third-degree relatives or Both parents (first-degree relatives)	2023	6	***18%***	high / very high
	2022	2		
	TOTAL	***8***		

The study reveals that there is a high risk of developing AD for patients with both parents affected by AD or for those who have one first-degree relative along with at least two second-degree or third-degree relatives with AD. This highlights the critical importance of genetic counseling and monitoring throughout an individual's life, including premarital, prenatal, and postnatal stages.

In the context of providing genetic counseling to patients from families with a history of chronic neurological conditions such as AD, prophylaxis primarily involves the implementation of programs for testing, early evaluation, and identification of both genetic and non-genetic risk factors, as well as individual predispositions and associated diseases that may exacerbate the vulnerabilities earlier. Additionally, it entails providing genetic counseling in accordance with the established protocols and specific guidelines for this complex medical procedure.

The process of providing genetic counseling and recommendations is grounded in the analysis of families or individuals, considering their level of education, training, and ability to comprehend the concepts presented. This includes defining and explaining anatomical processes and terminology. Additionally, the medical counselor must respect the individual's religious and ethical beliefs while addressing the specific characteristics of the identified condition (AD), including its severity and progression rates.

Conclusions

The purpose of the conducted study was to compile conclusive statistics, identify genetic factors, and correlate them with environmental ones, highlighting the importance of developing evaluation programs and early introduction of medication to decelerate the progression of neurodegenerative processes.

The analysis of the cohort emphasized the necessity to investigate known genetic mutations and to identify all genetic and biochemical alterations that may increase an individual's susceptibility to develop AD over the course of life. The familial predisposition can lead to the

irreversible progression of cognitive decline and the eventual loss of one's identity. Additionally, the study highlights the significance of exploring the complex interactions between genetic, physiological, demographic, and environmental factors that may insidiously trigger the inherited vulnerabilities. All the extensive familial investigations should be followed by the formulation and delivery of informed genetic counseling and recommendations.

The retrospective study conducted on patients admitted to the Neurology Department of the "Dr. Iacob Czihac" Clinical Military Emergency Hospital Iași highlights that AD occupies a significant position among chronic neurological disorders. Although the majority of patients do not present hereditary antecedents, predisposing diseases, environmental and behavioral factors, stress, as well as the region of residence, play fundamental roles in the onset of the disease. It is observed that individuals in the 60-70 age category, from urban areas, especially females, have a higher probability of developing AD.

In conclusion, with the global increase in incidence, prevalence, and mortality rates, focusing on identifying genetic and non-genetic factors involved in the development of neurodegenerative diseases, along with advancing research to create modern personalized therapies, is essential to counter these adverse trends. In this context, the medicine of the future is envisioned as both preventive and personalized.

Unequivocally characterized by a vast etiology, through cerebral atrophy associated with neurotrophin depletion, mitochondrial dysfunction, accumulation of neurofibrillary tangles, and senile plaques following the appearance of allelic variants (APOE4, MAPT, APOJ, SORT1) or dominant autosomal mutations (PSEN1/2, APP), AD is a multifactorial disorder resulting from the bilateral interaction between genetic and environmental factors or solely one of them. However, the transition from vulnerability to the actual disease is achieved through the continuous corroboration of these two major classes of factors.

References

Burns A, Byrne JE, Maurer K. 2002. Alzheimer's disease. Lancet. 360: 163–165. www.thelancet.com.

Gao Y, Liu X. 2021. Secular trends in the incidence of and mortality due to Alzheimer's disease and other forms of dementia in China From 1990 to 2019: An age-period-cohort study and joinpoint analysis. Front Aging Neurosci. 13(709156). doi:https://doi.org/10.3389/fnagi.2021.709156.

Gauthier S, Albert M, Fox N, Goedert M, Kivipelto M, Mestre-Ferrandiz J. 2016. Why has therapy development for dementia failed in the last two decades. Alzheimers Dement . 12: 60–64. doi:10.1016/j.jalz.2015.12.003.

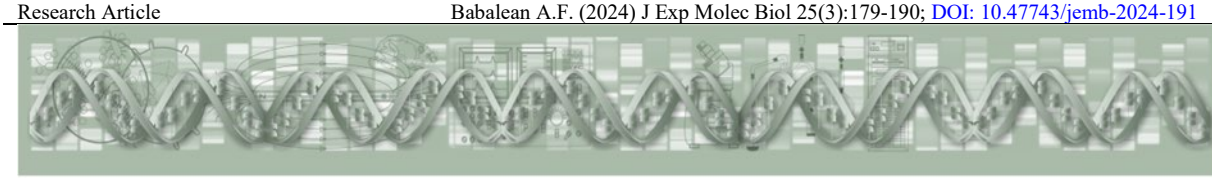
GBD 2019 Dementia Forecasting Collaborators. 2019. Estimation of the global prevalence of dementia in 2019 and forecasted prevalence in 2050: an analysis for the Global Burden of Disease Study 2019. Lancet Public Health. 7. doi:https://doi.org/10.1016/S2468-2667(21)00249-8.

GBD 2019 Diseases and Injuries Collaborators. 2020. Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet . 396: 1204–1222. doi:10.1016/S0140-6736(20)30925-9.

GBD 2019 Risk Factors Collaborators. 2020. Global burden of 87 risk factors in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet. 369: 1223–1249. doi:10.1016/S0140-6736(20)30752-2.

Gustavsson A, Norton N, Fast T, Frölich L, Georges J, Holzapfel D, Kirabali T, Krolak-Salmon P, Rossini PM, Ferretti MT, et al. 2023. Global estimates on the number of persons across the Alzheimer's disease continuum. Alzheimer's and Dementia. 19(2): 658–670. doi:10.1002/alz.12694.

Hippius H, Neundörfer G. 2003. The discovery of Alzheimer's disease. Dialogues Clin Neurosci. 5(1): 101–108. doi:10.31887/dcns.2003.5.1/hhippius.
Li X, Feng x., Sun X, Hou N, Han F, Liu Y. 2022. Global, regional, and national burden of Alzheimer's disease and other dementias, 1990–2019. Frontiers in Aging Neuroscience - Alzheimer's Disease and Related Dementias. doi:10.3389/fnagi.2022.937486. http://ghdx.
Livingston G, Huntley J, Sommerlad A, Ames D, Ballard C, Banerjee S. 2020. Dementia prevention, intervention, and care: 2020 report of the Lancet Commission. Lancet . 396: 413–446. doi:10.1016/S0140-6736(20)30367-6.
Masters CL, Bateman R, Blennow K, Rowe CC, Sperling RA, Cummings JL. 2015. Alzheimer's disease. Nat Rev Dis Primers. 1. doi:10.1038/nrdp.2015.56.
Maurer K, Volk S, Gerbaldo H. 1997. Auguste D and Alzheimer's disease. THE LANCET. 349: 1546–1549.
Soria Lopez JA, González HM, Léger GC. 2019. Alzheimer's disease. In: Handbook of Clinical Neurology. Vol. 167. Elsevier B.V. p. 231–255.
Stănescu A. 2015. Totul despre Alzheimer. Târgu Mureș: Farmamedia.
Szalontay AS. 2014. Actualități în boala Alzheimer. Iași: Editura Gr. T. Popa.
Szalontay AS, Chiriță V, Chiriță R. 2005. Boala Alzheimer - Management Clinico-Terapeutic. Iași: Editura U.M.F. "Gr.T.Popa" .
Tiwari S, Atluri V, Kaushik A, Yndart A, Nair M. 2019. Alzheimer's disease: Pathogenesis, diagnostics, and therapeutics. Int J Nanomedicine. 14: 5541–5554. doi:10.2147/IJN.S200490.



A MORPHOLOGICAL AND BIOLOGICAL ASSESSMENT OF *CRENOBIA ALPINA* AND *CRENOBIA MONTENIGRINA* (PLATYHELMINTHES, TRICLADIDA, PLANARIIDAE) IN THE PARÂNG MOUNTAINS (ROMANIA)

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Abstract

The paper analyses the morphology of the copulatory apparatus and the reproductive isolation of the monopharyngeal and polypharyngeal *Crenobia* (Dana, 1766) in sympatric populations of the Parâng Mountains, Romania. The copulatory apparatus of the two forms shows a non-simultaneous development, indicating a seasonal reproductive isolation: the monopharyngeal *Crenobia alpina* (Dana, 1766) becomes able to mate during the warm season while the polypharyngeal *Crenobia montenigrina* (Mrázek, 1904) becomes able to mate during the cold season. The mature copulatory apparatus of the monopharyngeals and the polypharyngeals shows all the characters typical for *C. alpina*, respectively for *C. montenigrina* as presented in the literature. In both species, some specimens reveal a complex system of fine ducts / sclerotized lines into the wall of the genital atrium, which are attributed to biological structures of unknown function rather than histological artefacts.
Keywords: *Crenobia*, monopharyngeal, polypharyngeal, reproductive isolation

Introduction

Crenobia is a genus of freshwater flatworm with a limited dispersal ability which lives almost exclusively in cold springs and headwaters of mountainous areas in Europe and Turkey (Brändle et al. 2007, Brändle et al. 2017, Sluys 2022).

Taxonomically, *Crenobia* is a group of species (*Crenobia alpina* sensu lato) with different morphology, reproductive biology, karyology, ecological requirements, habitat, geographic distribution (Sluys 2022). The various forms of this complex were regarded either as distinct species within the genus *Crenobia* or as subspecies of *Crenobia alpina* (see the synthetic review of Sluys, 2022).

Regardless of whether they are species or subspecies, the genus *Crenobia* comprises two distinct morphological types in terms of the number of pharynges:
a) the monopharyngeal type includes *Crenobia alpina septentrionalis* (Thienemann, 1938), *Crenobia alpina meridionalis* [= *Crenobia alpina alpina*], *Crenobia alpina alba* (van Oye, 1935), *Crenobia alpina bathycola* (Steinmann, 1911), *Crenobia alpina corsica* (Arndt, 1922)
b) the polypharyngeal type includes *Crenobia alpina montenigrina* with 10 -15 pharynxes, *Crenobia alpina anophthalma* (Mrázek, 1907) with 3 pharynxes, *Crenobia alpina teratophila* (Steinmann, 1908) very similar with *C. montenigrina* and a form with 24 – 35 pharynxes from Bulgaria, described by Chichkoff as *Phagocata cornuta* (Codreanu 1956, Sluys 2022).

Received 3 April 2024; Revised 2 September 2024; Accepted 14 September 2024;
Published Online 17 September 2024; Published 9 October 2024

The forms present in the Romanian fauna are the monopharyngeal *C. alpina* and the polypharyngeal *C. montenigrina* (Babalean 2020, Codreanu 1956, Sluys 2022). In the Parâng Mountains the two forms occur sympatrically in mixed populations (Babalean 2020). Codreanu (1956) indicates the seasonal reproductive isolation of *C. alpina* and *C. montenigrina*. The copulatory apparatus of the two forms is very similar; the minor differences between them should not prevent mating. The two morphological types are discussed from the biological species concept, of reproductive isolation, according to literature (Bănărescu 1973, De Queiroz 2007).

The aim of the paper is to assess and verify the seasonal reproductive isolation of the monopharyngeals and polypharyngeals, on morphological grounds.

Materials and Methods

The study is based on more than 300 specimens sampled between 1.07.2016 and 7.11.2022 from Rânca Resort – the Parâng Mountains. There were chosen several locations (Fig. 1) with different habitats: three reocrene springs (CB-Romanul, 45°17′51.83″N / 23°40′27.30″E), springs with sandy reservoirs and their collecting streams (Tidvele area, 45°19′52.32″N / 23°42′ 00.62″E, Paltinul Mountain area, 45°18′45.98″N / 23°40′01.08″E), springs modified by human activity – spring with collecting pipe and reservoir Păpușa Peak, (45°19′19.82″N / 23°41′14.22″E), mountain stream (ski lift area 45°18′48.75″N / 23°41′08.32″E). The sampling was done during summer (June, July) and during the cold season (November). To avoid defaunation, only a 1/4th to maximum a half of the visible specimens were collected from most identified populations; in only one location, all the visible specimens (1-3-5) of the population were collected.

Part of the worms was fixed in Beauchamp solution for 24 hours, thereafter, removed and stored in ethanol 75º; another part of the worms was fixed and stored in 96º ethanol. The maturity of the copulatory area was assessed on histological sections and by visual inspection of fixed, uncut worms. In this latter case, the specimens with large copulatory area were considered capable for the sperm transfer during mating. Some specimens were processed by usual histological techniques for Haematoxilin-Eosin staining, for histological frontal and sagittal serial sections at 5 µm thick:

- **monopharyngeal** specimens: CR1, Cm1, CR4, Cm2 – sexually mature, collected in summer: CR1 – (CB-Romanul), sagittal sections on 53 slides; Cm1 – (Paltinul Mountain), sagittal sections on 63 slides; CR4 – (CB-Romanul), frontal sections on 12 slides; Cm2 – (CB-Romanul), transversal sections on 25 slides
- **polypharyngeal** specimens: CR2, CR3, Cp1, Cp2 – immatures, collected in summer; CR8 – sexually mature, collected in November.

CR2 – (CB-Romanul), sagittal sections on 43 slides; CR3 – (Tidvele area), frontal sections on 10 slides; Cp1 – (Tidvele area), sagittal sections on 40 slides; Cp2 – (Tidvele at the highest altitude – 1841 m), frontal sections on 9 slides; CR8 – (Păpușa spring), sagittal sections on 71 slides.

Abbreviations used in the figures: af – atrial fold, bc – bursal canal, cb – copulatory bursa, cov – common oviduct, da – distal atrium, fa – fibrous atrium (fibrous part of the atrium), fd – fine ducts, go – gonopore, ma – muscular part of the atrium, mo – mouth, ov – oviduct, ph – pharynx, pp – penis papilla, vd – vas deferens.

Results and discussions

Results

1. Types of populations

In the studied area, *C. alpina* and *C. montenigrina* formed the following types of populations with respect to the pharyngeal condition (mono- / polypharyngeal) and the number of collected specimens:

A) non-mixed populations

a) exclusively monopharyngeals, with a small number of specimens – only one monopharyngeal population (ski lift area)

b) large populations, exclusively polypharyngeals – springs and some collecting streams

B) mixed populations:

a) predominant monopharyngeals – collecting stream at the Paltinul Mountain base

b) predominant polypharyngeals – some collecting streams

c) nearly equal proportions of mono- and polypahryngeals – reocrene no. 3 (CB-Romanul)

Some non-mixed populations were identified as mixed at the next collection (next years) and in the different seasons (warm season – cold season) - reocrene no. 2 (CB-Romanul). However, the existence of non-mixed populations exclusively monopharyngeals or exclusively polypharingeals is uncertain because no population was sampled completely.

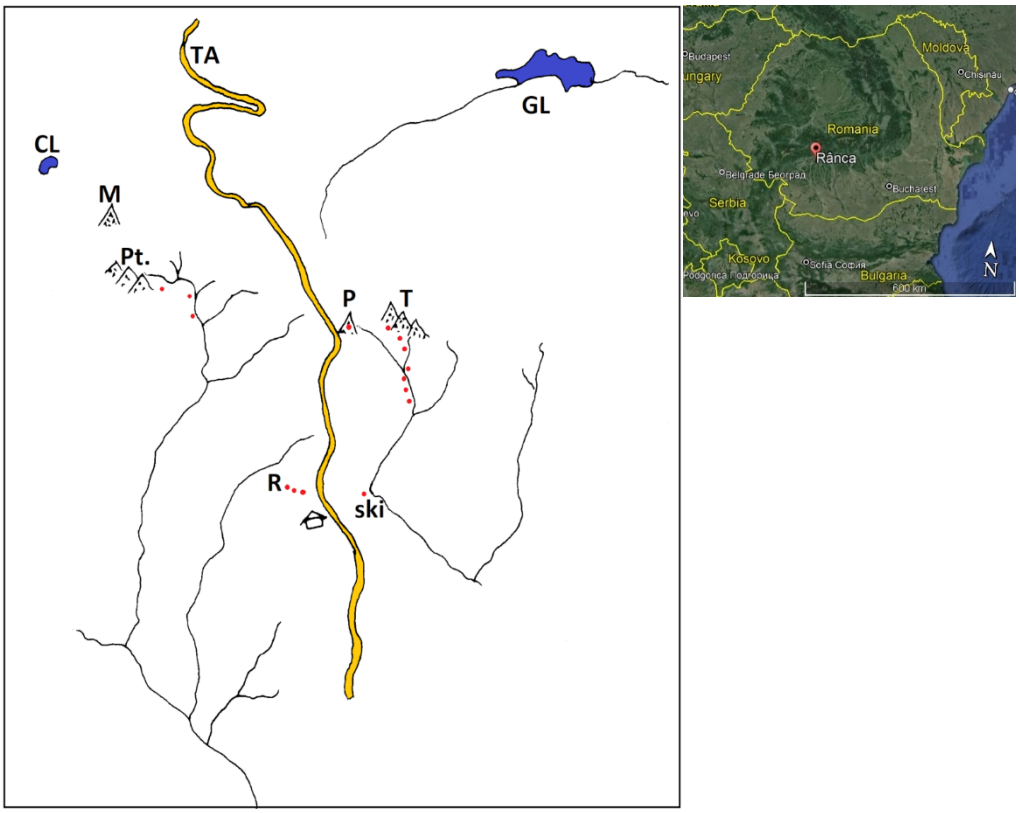

Figure 1. Location of the collecting site: Rânca, the Parâng Mountains, Romania (inset - Google Earth Pro map of Romania showing the location of the Parang Mountains); sampling points are indicated as red dots, CL – Câlcescu Lake, TA – Transalpina road, GL – Galbenul Lake, M – Mohorul Mountain, Pt. – Paltinul area, Păpușa peak, T – Tidvele area, R – CB-Romanul area with reocrene springs, ski - ski lift area (redrawn and adapted from Popescu (1986))

2. Morphology

The monopharyngeal *Crenobia alpina* (specimens CR1, Cm1, Cr4, Cm2)

These four specimens were collected in summer and are sexually mature.

The mouth opening is located at the posterior end of the pharyngeal pocket.

With very few exceptions these specimens reveal ovaries, few prepharyngeal ventral follicular testes, ducts and fully developed copulatory apparatus. The exceptions are attributed to a

physiological state (the lack of ovaries and oviducts in specimen CR1 is considered a physiological state / a stage of the reproductive cycle).

The copulatory apparatus (Figs. 2-6).

The copulatory apparatus match the patterns of the genus *Crenobia* and *C. alpina*:

- an oversized (well developed) atrium consisting of 2 distinct parts: (i) the external muscular part, very thick, composed by muscular plates (bundles) in radial disposition, surrounded by an outer zone of myoblasts (Sluys 2022) and (ii) the internal fibrous layer
- the penis has an indistinct bulb and a long papilla, extending beyond the atrial fold – Fig. 3
- the genital atrium is divided by an atrial fold in 2 regions: (i) the proximal region which houses the most part of the penis papilla; it also receives the common oviduct and the bursal canal (Sluys 2022) and (ii) a distal region which opens by the gonopore (the genital orifice).
- the two vasa deferentia (spermiducts) with large spermiducal vesicles in their terminal part, at a certain distance before opening into the penis bulb.

Besides the above general characters, the histological slides of the four analysed specimens reveal some peculiarities: the course of the two vasa deferentia is symmetric in specimens CR1 and CR4 and asymmetric in specimen Cm1; the presence of a complex system of apparently fine ducts or sclerotized lines in the wall of the genital atrium (Figs. 2-4). These structures seem to be arranged between the atrial space housing the penis papilla, the distal atrium, and the penis bulb, interconnecting them. Orifices corresponding to the fine ducts are visible in the penis bulb and in the wall of the distal atrium.

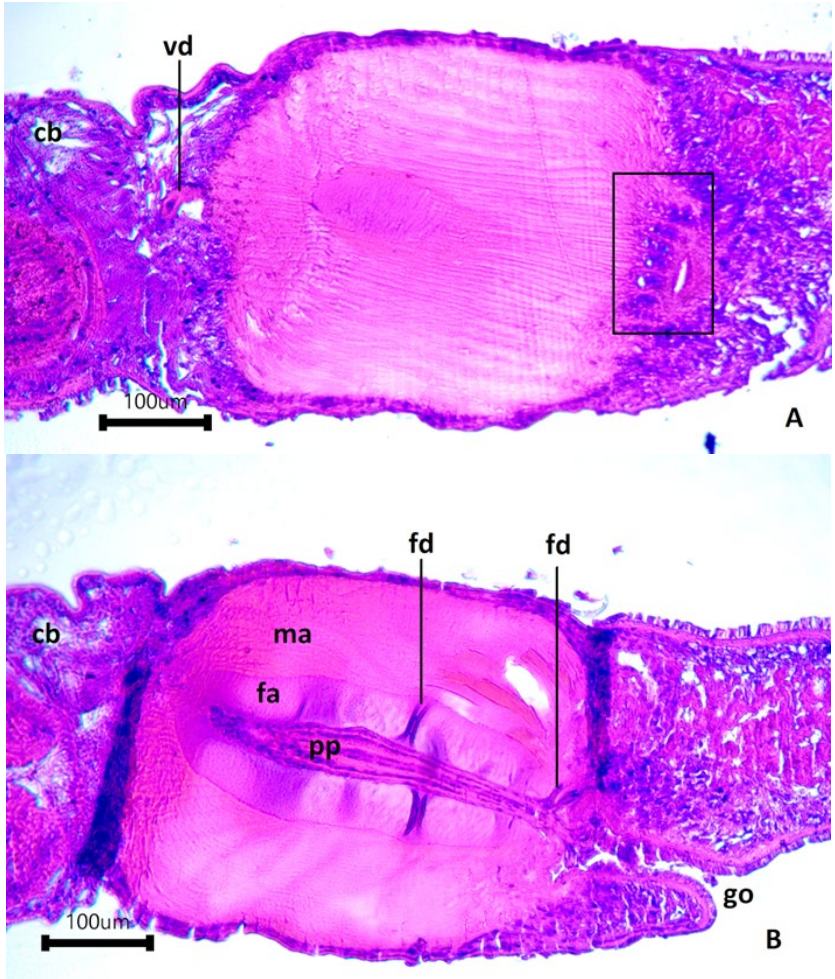

Figure 2. Microphotographs of the copulatory apparatus of specimen CR1 (monopharyngeal), sagittal sections: A – slide CR1-22-2 showing the orifices corresponding to the fine ducts (in square); B – slide CR1-25-1

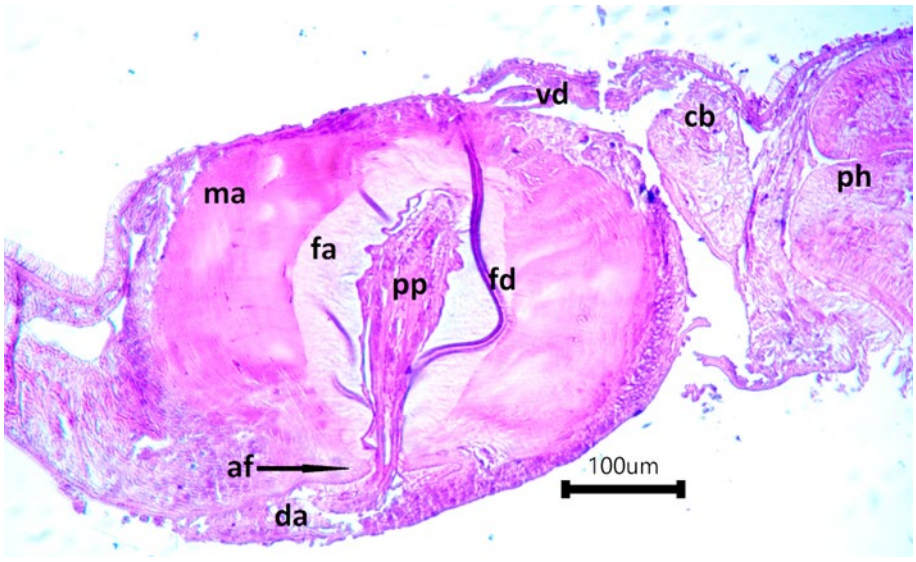

Figure 3. Microphotograph of the copulatory apparatus in specimen Cm1 (monopharyngeal), sagittal sections, slide Cm1-33-3

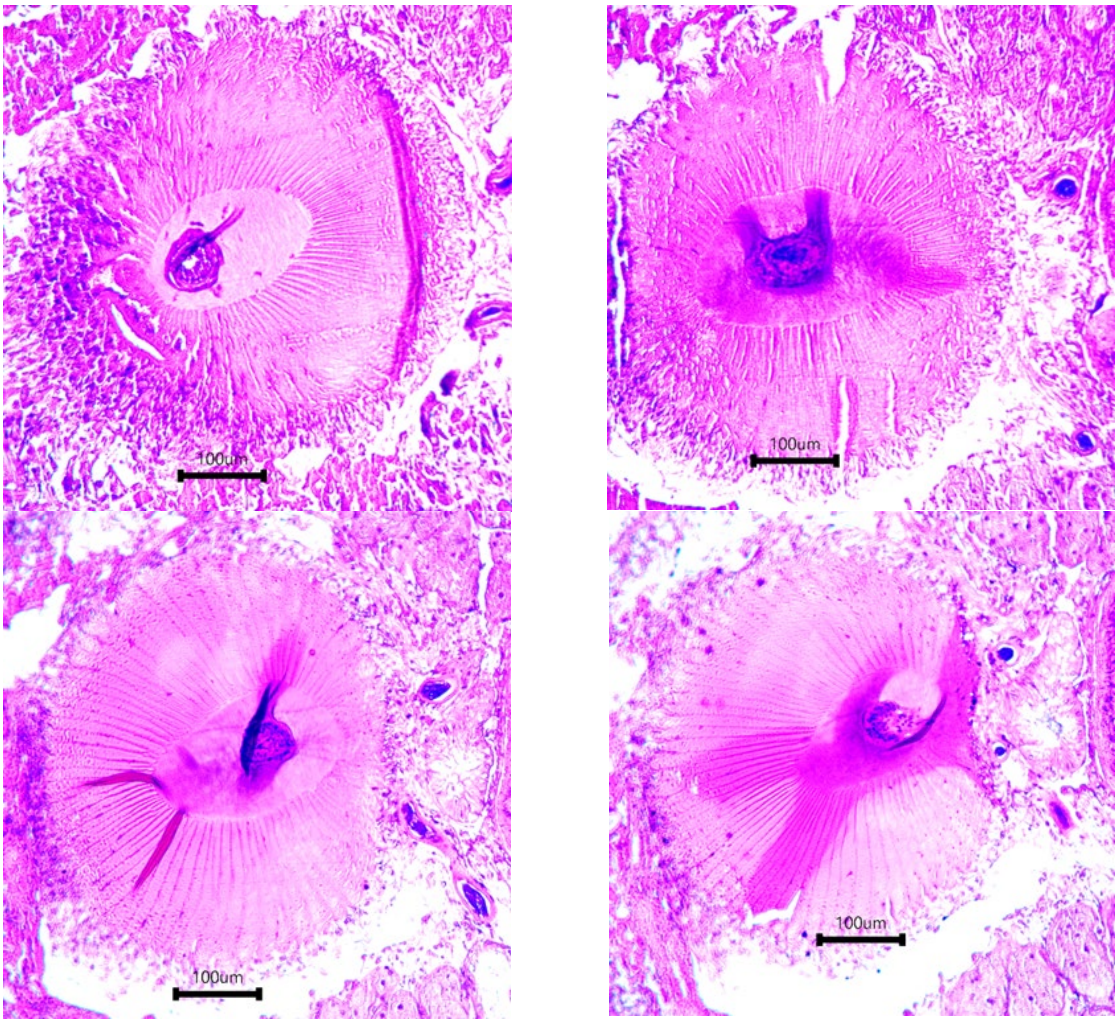

Figure 4. Microphotographs of the genital atrium in specimen CR4 (monopharyngeal), the muscular and fibrous regions showing fine ducts / sclerotized lines

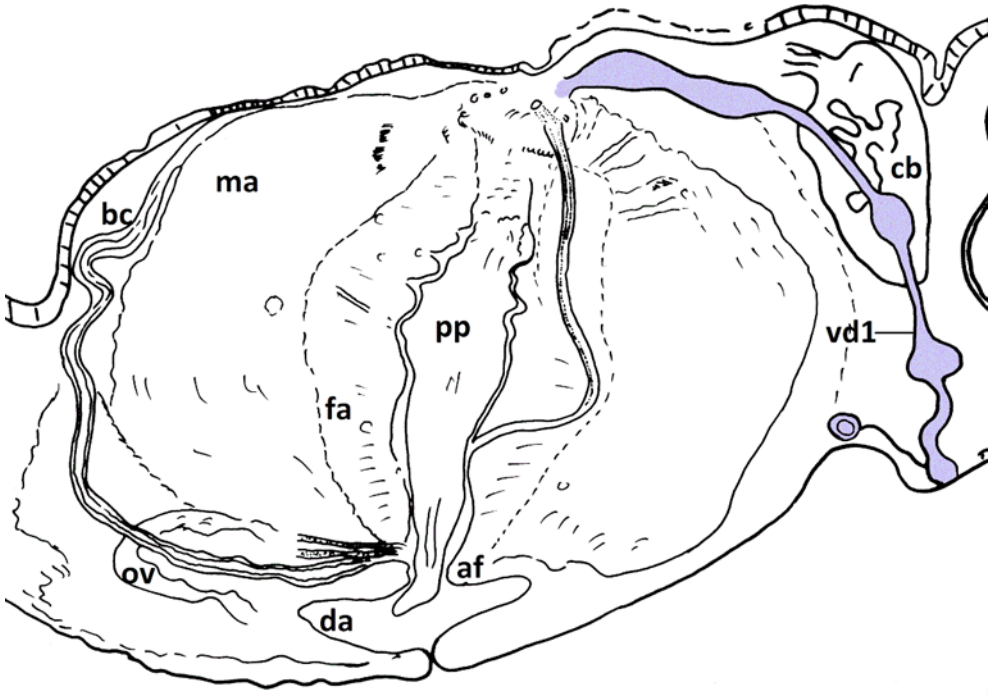

Figure 5. *Crenobia alpina* (monopharingeal, specimen Cm1), sagittal reconstruction of the copulatory apparatus with the course of the right vas deferens, anterior to the right

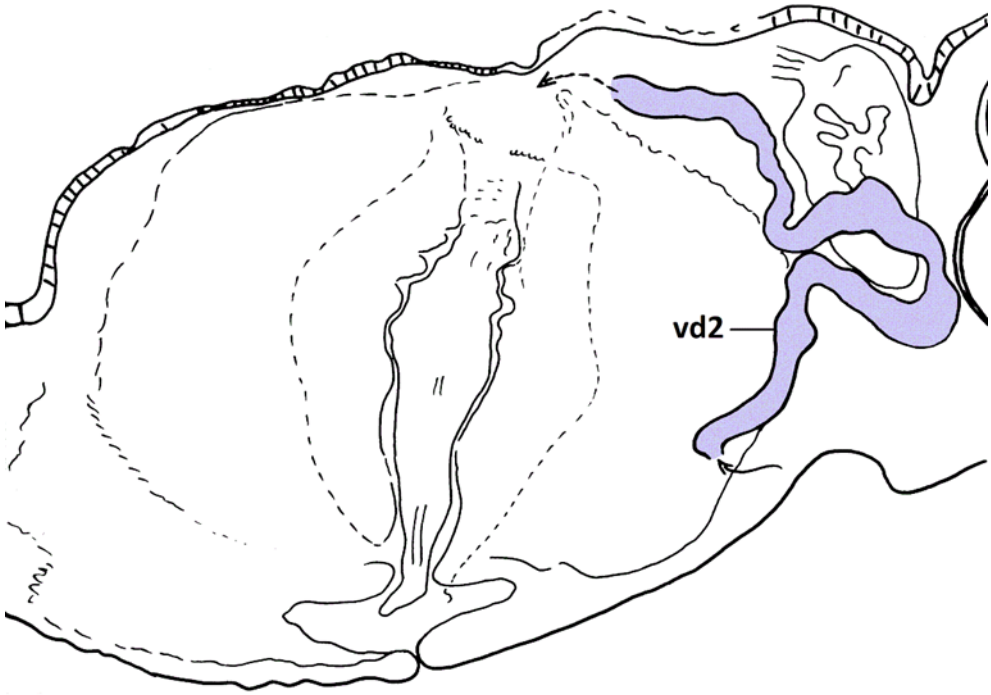

Figure 6. *Crenobia alpina* (monopharingeal, specimen Cm1), sagittal reconstruction of the copulatory apparatus with the course of the left vas deferens, anterior to the right

The polypharyngeal *Crenobia montenigrina* (CR8 – sexually mature, collected in November; CR2, CR3, Cp1, Cp2 – immature individuals, collected in summer)

Besides the pigmented specimens, unpigmented (white) and marbled polypharyngeals were also collected (Fig. S1). Numerous pharynxes, opening into a common space with a posterior mouth, at a certain distance from the genital orifice (shifted anteriad) (Fig. 7).

The mature specimen CR8 show small and rare pre-pharyngeal follicular testes located ventrally. The copulatory apparatus (Figs. 7, 8) presents most of the characteristics of *C. montenigrina* as described in the literature (Sluys 2022), excepting the asymmetry of the vasa deferentia. The fine ducts / sclerotized lines are present but to a much lesser extent than in the monopharyngeals.

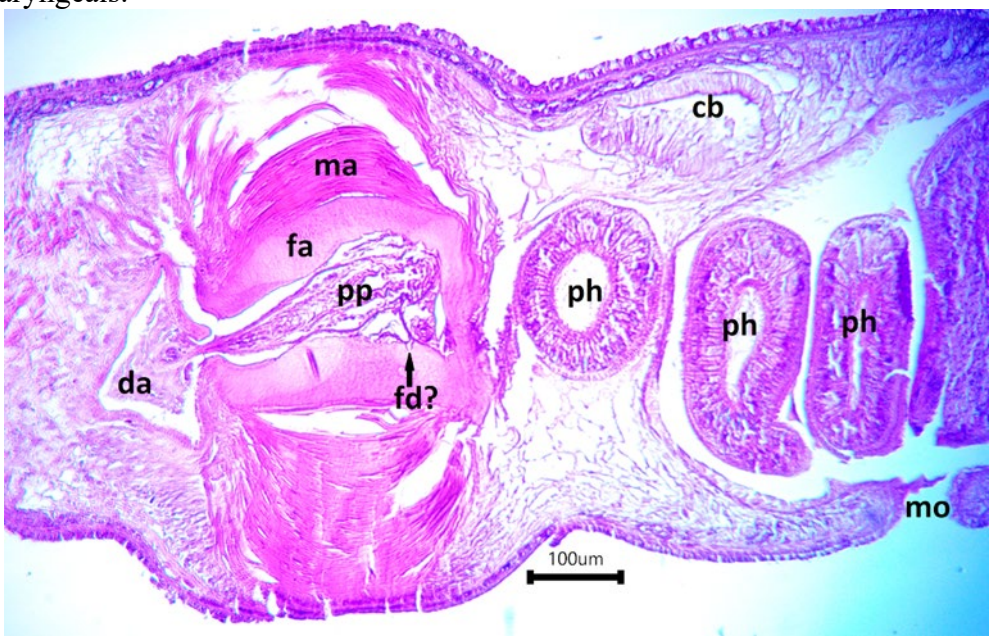

Figure 7. Microphotographs of the copulatory apparatus in specimen CR8 (polypharyngeal), sagittal sections, slide CR8-39-3

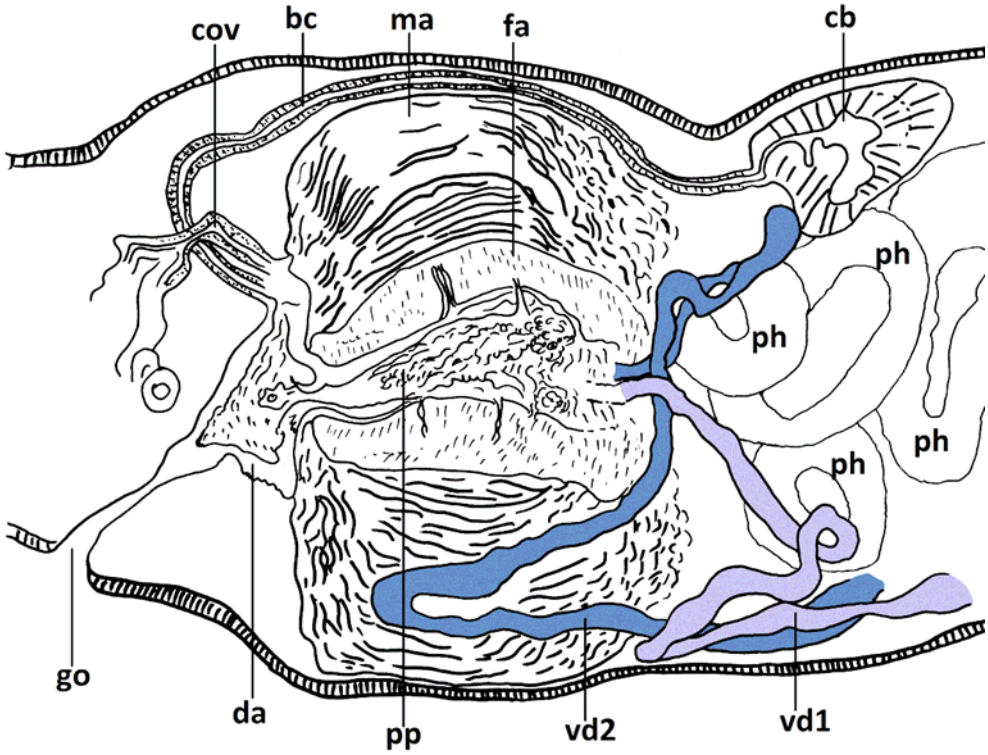

Figure 8. *Crenobia montenigrina* (polypharyngeal, specimen CR8), sagittal reconstruction of the copulatory apparatus, anterior to the right

The immature polypharyngeal specimens (sampled in summer) reveal incompletely developed copulatory apparatus, developing follicular testes and ovaries on histological sections (Fig. S2).

Morphologically, the Romanian specimens of both *Crenobia alpina* and *Crenobia montenigrina* fit quite well the description from literature (Sluys 2022). Minor differences such the symmetry of the vasa deferens in specimen CR1 (monopharyngeal) and the asymmetry in specimen CR8 (polypharyngeal) might be individual characters (intrapopulational variability).

A comparison of the morphological characters analysed in this paper for the two species is presented in Table 1.

Table 1. A synthetic comparison between *C. alpina* s. str. and *C. montenigrina*

Character	*C. alpina* s. str.	*C. montenigrina*
Number of pharynxes	One pharynx	Numerous
Mouth opening	At the posterior end of the pharyngeal pocket	Shifted anteriad
Testes position	Prepharyngeal, ventrally	Prepharyngeal, ventrally
Vasa deferens trajectory; the loops	Symmetrical and asymmetrical	Asymmetrical and symmetrical (literature)
Penis papilla	Extends beyond the atrial constriction – Fig. 3	Extends beyond the atrial constriction – Fig. 7

The characters with diagnostic value appear to be the number of pharynxes and the position of the mouth opening; the two species appear to be cryptic at the level of the copulatory apparatus.

The complex system of fine ducts / sclerotized lines are possibly genuine biological elements involved for instance in shedding and releasing cocoons.

3. Aspects regarding the reproductive biology (Figs. 9, 10; Table 2)

The monopharyngeal *Crenobia alpina*

During the warm season (June, July), the following types of monopharyngeals were collected: mostly specimens with large, well-developed copulatory area (Fig. 9Aa); two specimens with a dilated, oversized copulatory area having the aspect of an internal cocoon (Fig. 9 Ab); a few specimens with small copulatory area; a few asexual specimens, without copulatory area. External cocoons were not observed.

During the cold season (November), most part of the monopharyngeals show small copulatory area, a smaller proportion are asexual without copulatory area. The asexuals have a distinct rounded appearance of the posterior end (Fig. 9B**)**. The rounded posterior end is here associated with hatching, such specimens resulted by hatching.

Fission not observed. Mating not observed.

The polypharyngeal *Crenobia montenigrina*

During the warm season (June, July) most specimens have small copulatory area, and some are asexual (they lack the copulatory area).

During the cold season (November), all the specimens in most springs presented large copulatory area; in only one investigated spring, all specimens were asexuals with no copulatory area – reocrene no. 2 (CB-Romanul), November 2022.

Neither external, nor internal cocoons were observed. The polypharyngeals were found undergoing fission during July and November in non-mixed populations (for instance reocrene

spring no. 2 – July 2021, Păpușa spring – November 2020 (Fig. 10 A, B). Specimens most probably resulted from fission were sampled in November (Tidvele area); their rear end has a tapering appearance (Fig. 10C).

The population of the reocrene spring no. 2 (CB-Romanul) consisted of only immatures polypharyngeals on 05 July 2021, and as a mixture of 7 asexual polypharyngeals and one asexual monopharyngeal on 07 November 2022. In this latter case of mixed population, polypharyngeals and monopharyngeals cannot interbreed due to their immaturity. This spring was found almost completely deteriorated in November 2022.

Mating not observed in polypharyngeals.

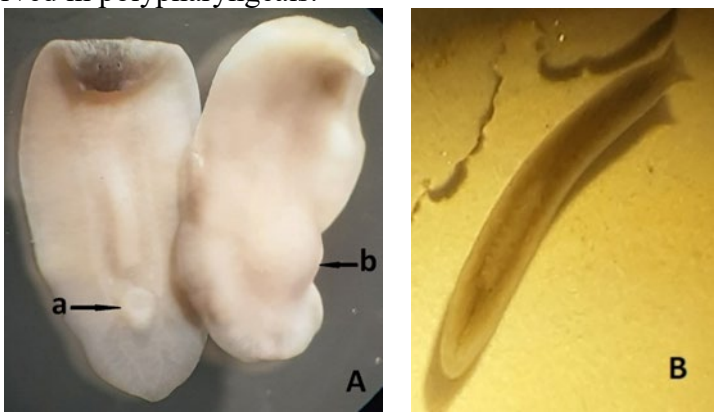

Figure 9. *Crenobia alpina*, general habitus: A – in summer, a – large copulatory area, b – oversized copulatory area; B – in November, small asexual specimen with rounded posterior end, most probably resulted from the late summer /early autumn hatching.

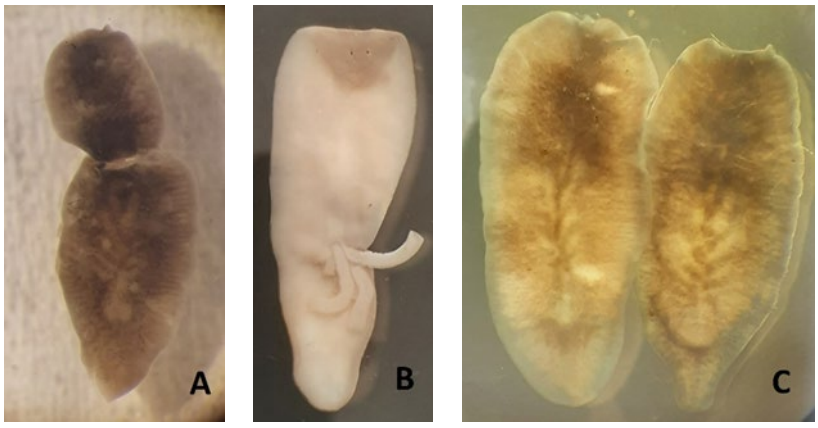

Figure 10. *Crenobia montenigrina* general habitus: A – specimen undergoing fission (5 November 2020), B – specimen undergoing fission (5 July 2021), C – specimen (the right specimen) with the rear end tapered, most probably resulted from fission

The reproductive characteristics of the monopharyngeals and polypharyngeals are synthesized in Table 2.

Table 2. Comparative synthetic table of the seasonal reproductive characteristics of the monopharyngeal *C. alpina* and the polypharyngeal *C. montenigrina*

	Copulatory area/reproductive biology characters Warm season	Copulatory area/reproductive biology characters Cold season
Monopharyngeal specimens *C. alpina*	- large copulatory area in most specimens	- small copulatory area - asexuals (copulatory area absent)

	- internal cocoons (copulatory areas with the aspect of internal cocoon) - fission not observed	- fission not observed
Polypharyngeal specimens *C. montenigrina*	- small copulatory area - absent copulatory area - undergo fission	- large copulatory area - no cocoons observed - undergo fission

A schematic biological cycle can be sketched based on the observed reproductive characters:

The monopharyngeals mate and produce cocoons (internal, maybe external as well) during the warm season (June, July). The few asexuals or those with small copulatory area are most probably resulted from cocoons hatching. These hatchlings grow and start to develop the copulatory area. They become specimens with small copulatory area, unable for sperm transfer in the cold season (November). The November asexuals (with no copulatory area) result from late summer /early autumn hatching.

The polypharyngeals mate during the cold season when they have completely developed copulatory area. They are unable to mate during the warm season, as they have small copulatory area or lack it.

This study strongly indicates the seasonal reproductive isolation of *C. alpina* and *C. montenigrina*. A deeper and true reproductive isolation should be verified by the comparative study of spermatozoa in the two forms.

The natural history of *C. alpina* and *C. montenigrina*, much discussed in the literature (Codreanu 1956 and reference therein) remains still unsolved and raises many questions: When and how the two species appeared? Why do they form sympatric populations in the Carpathian (Parâng) Mountains? Are they sympatric elsewhere in Europe?

As to their origin, three possibilities can be discussed:

1) *C. alpina* and *C. montenigrina* have a common ancestor which disappeared from the current fauna. In this hypothesis, from a common pre-glacial ancestor (before the Quaternary Glaciation) resulted sympatric *C. alpina* and *C. montenigrina* - with a much northern geographical distribution, later modified by the glaciation. Both species are cryophilic, but regarding the reproductive period, *C. montenegrina* is more cryophilic (sexually mature in the cold season). In the cold periods of the Glaciation (the glaciation stadials) both species would have undergone a southward migration. *C. montenigrina* settled in high mountainous areas, corresponding to their thermal optimum. In warm periods (interstadials), *C. alpina* would have undergone the reverse migration path from south to north while *C. montenigrina* did not.

2) *C. montenigrina* resulted from *C. alpina*. This theory must admit the present geographical range of *C. montenigrina* as speciation center. A question that needs to be answered is: How the pharyngeal number (one / numerous) is related to shifting reproductive season?

3) *C. alpina* resulted from *C. montenigrina* – for the moment this hypothesis is difficult to be accepted.

Conclusions

The present results are consistent with the theory of temporal reproductive isolation of the two species, expressed by Codreanu (1956). *C. alpina* and *C. montenigrina* remain species of great interest for future studies. The factors that influence the reproductive biology of the two species in sympatry are poorly understood.

Acknowledgments: This work has received no fund. I thank the anonymous reviewers for the recommendations to improve the paper.

Supplementary figures

Figure S1. *Crenobia montenigrina* - black, unpigmented and marbled living specimens

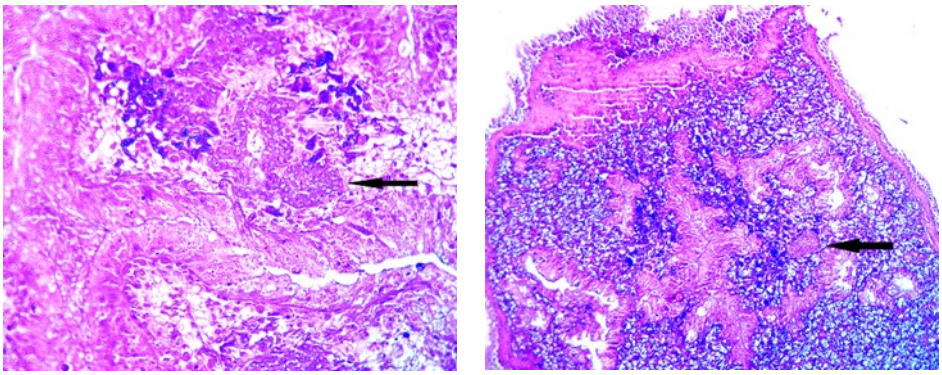

Figure S2. Developing follicular testes (left) and ovary (right) in immature polypharyngeal specimen CR3 (scale bar not available)

References

Babalean AF. 2020. Contributions to Platyhelminthes – Tricladida and Arachnida – Opiliones, Sitech. Craiova.

Bănărescu P. 1973. Principiile și metodele zoologiei sistematice. Editura Academiei R.P.R.

Brändle M, Heuser R, Marten A, Brandl R. 2007. Population structure of the freshwater flatworm *Crenobia alpina* (Dana): old lineages and low gene flow. Journal of Biogeography. 34:1183-1192. https://doi.org/10.1111/j.1365-2699.2007.01689.x

Brändle M, Sauer J, Opgenoorth L, Brandl R. 2017. Genetic diversity in the Alpine flatworm *Crenobia alpina*. Web Ecology. https://doi.org/10.5194/we-17-29-2017

Codreanu R. 1956. Răspîndirea unei planarii polifaringiene (*Crenobia alpina montenigrina* / Mrazek / 1903 în Carpații Sudici. Analele Institutului de Cercetări Piscicole. vol. I:385-399.

De Queiroz K. 2007. Species Concepts and Species Delimitation. Systematic Biology. 56(6): 879-886. https://doi.org/10.1080/10635150701701083

Popescu N. 1986. Parâng, Colecția Munții noștri. Editura Sport Turism București.

Sluys R. 2022. Biogeography and diversification of the freshwater planarian *Crenobia alpina* sensu lato (Platyhelminthes, Tricladida): A synthetic review. Zoologischer Anzeiger. 300:12-26. https://doi.org/10.1016/j.jcz.2022.05.010