UNIVERSITY OF WARMIA AND MAZURY IN OLSZTYN

Polish Journal of Natural Sciences

39

(1/2024)

PUBLISHER UWM
OLSZTYN 2024

The Polish Journal of Natural Sciences is indexed and abstracted
in Biological Abstracts and Biosis Previews

The print edition is the primary version of the Journal

The Journal is also available in electronic form on the websites
http://www.uwm.edu.pl/polish-journal/ (home page)
https://czasopisma.uwm.edu.pl/index.php/pjns/about (electronic platform; submissions)

PL ISSN 1643-9953

Publisher UWM Olsztyn
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10-718 Olsztyn-Kortowo, Poland
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Edition 56 copies; publisher's sheets 5.7; number of printed sheets 4.75
Print Zakład Poligraficzny UWM w Olsztynie
order number 709

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Biology and Forestry

POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 39(1): 5–14, Y. 2024

DOI: 10.31648/pjns.9770

APOPTOSIS OF NEUTROPHILS, MONOCYTES, AND LYMPHOCYTES IN THE PERIPHERAL BLOOD OF COWS DURING LACTATION

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Key words: Cattle (*Bos taurus*), lactation, apoptosis, immune system.

Abstract

The immune system of animals plays an important role in the homeostasis system. The aim of the study was to detect apoptosis of immune cells in the peripheral blood stream at different periods of lactation in cows. The investigation of the immune defense of cows was conducted during various lactation periods: early (3–5 days), mid (90–150 days), and late (drying off – 5–7 days; dry period – 12–20 days) periods of mammary gland function. The highest intensity of apoptosis was observed in neutrophils and monocytes during the mid-lactation period. The highest intensity of apoptosis is observed in phagocytic cells during the colostrum secretion period and mammary gland involution. The study of physiological constants of blood cell apoptosis will serve as a basis for developing informative methods of mastitis diagnosis in cows and implementing effective treatment measures. The findings obtained from this research are valuable for practical veterinarians and from a public health perspective.

Introduction

Currently, there is active research worldwide on measures for preventing cow mastitis. It is important to emphasize that some researchers draw parallels between non-specific immunobiological reactivity and resistance, constantly exploring innovative ways of molecularly regulating immune response, including apoptosis of immune cells. This includes investigating the role of various immunomodulators, such as cytokines, growth factors, and signaling molecules, in enhancing the immune system's ability to resist mastitis-causing pathogens (LIU 2018, CVETNIC et al. 2021). Researchers are exploring novel vaccination strategies, probiotics, and nutraceuticals that can enhance the cow's natural defenses against

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mastitis. These efforts underscore the ongoing commitment to developing effective and sustainable methods for preventing this prevalent disease in dairy cattle (Burton et al. 2003, Lim et al. 2017, Zhelavskyi 2021).

The process of apoptosis is the outcome of diverse factors that contribute to cell death. These factors may encompass nonspecific elements like temperature fluctuations, exposure to toxic substances, oxidants, free radicals, as well as γ and ultraviolet radiation, along with bacterial toxins. In all of these cases, apoptosis is induced, but with increasing impact of the respective agent, necrotic cell death is initiated (Wang et al. 2019, Cvetnic et al. 2021), which has sparked increased interest among researchers, particularly in hormonal regulation of apoptosis (Wynn and Vannella 2016, Kydonaki et al. 2021, Wang et al. 2020, Zhelavskyi et al. 2021).

The initiation of apoptosis is caused by physiological signal-inducing molecules that are recognized by specialized cellular receptors, triggering a cascade of subsequent intracellular biochemical processes (Zhou et al. 2015, Bae et al. 2022). These signals can originate from various factors, including biologically active substances, hormonal imbalances, antigen overload, the presence of specific antibodies to cell receptors, cytokines, and others (Psaila et al. 2016, Schnabel et al. 2020). Apoptosis can be prevented or regulated by various factors, including biological molecules that promote or inhibit apoptosis, as well as regulatory intracellular mechanisms (Levin et al. 2016, Kim et al. 2021, Zhelavskyi et al. 2024). Considering that apoptosis is a general biological mechanism of regulation and balance, responsible for maintaining the physiological balance of cell populations and eliminating distorted, mutated, and defective cells, new approaches are being considered for the treatment and prevention of diseases.

Therefore, this study aimed to determine the process of physiological aging and cell death in the peripheral bloodstream at different stages of lactation in cows.

Materials and Methods

Animals' criteria. A total of 112 cows (*Bos Taurus*, Ukrainian black-and-white milk breeding) ranging from 3 to 5 years of age were selected for an experiment. Cows were divided into four groups. Each group consists of 28 animals. The first group cows in the period of colostrum secretion (3–5 days); the second – cows in the middle lactation period (90–150 days); the third – drying off (5–7th day) and the fourth group – in the dry period (12–20th day). All animals were clinically healthy and belonged to the

farms of the Khmelnytskyi and Vinnytsia regions of Ukraine. Blood samples were taken from animals between 7:00 and 9:00 in the morning. Blood was taken from *v. jugularis* into vacuum-sealed glass containers.

This investigation was approved according to the Law of Ukraine "On the Protection of Animals from Cruel Treatment" (No. 3447-IV of February 21, 2006) and according to the requirements of the European Convention for the Protection of Pet Animals (ETS No. 125, Strasbourg, 13/11/1987). All experiments were carried out with the Ethical Permit at the Vinnytsia National Agrarian University, Ukraine. All animal manipulations were performed by the European Convention for the Protection of Vertebrate Animals and used for experimental and scientific purposes (Strasbourg, 18 March 1986).

Apoptosis analysis and detection

A blood sample was collected into a tube containing an anticoagulant Ethylenediaminetetraacetic acid (EDTA, Maxwell®, USA), and it was mixed gently to prevent clotting. The volume of Lymphoprep needed was calculated based on the amount of blood and desired cell yield, ratio of 1:1 (volume of blood to volume of Lymphoprep, Axis Shield Poc AS, Oslo, Norway) was used. The calculated volume of Lymphoprep was layered at the bottom of a centrifuge tube using a pipette. The whole blood sample was carefully layered on top of the Lymphoprep solution using a slow and gentle pouring technique to avoid disturbing the layers. The tube was centrifuged at specific settings optimized for cell separation. The recommended conditions for most blood cell isolations were: centrifugation at 800–1000 x g for 20–30 minutes at room temperature (20–25°C) without braking. A swing-out rotor was used if available to prevent mixing of layers during centrifugation. After centrifugation, three distinct layers were visible in the tube: the top layer containing plasma and lymphocytes, the middle layer containing monocytes, and the bottom layer containing granulocytes, including neutrophils.

Each layer was carefully aspirated and transferred into separate tubes using a Pasteur pipette or a pipetting device. The top layer (lymphocytes) was transferred into one tube, the middle layer (monocytes) into another tube, and the bottom layer (neutrophils and granulocytes) into a third tube. Each cell population was washed with Phosphate-buffered saline (PBS, pH 7.4; Sigma-Aldrich, USA) or a suitable buffer to remove Lymphoprep and other contaminants. The cells were then centrifuged at a low speed (300 x g) for 10 minutes to pellet the cells. The supernatant was discarded, and the cell pellets were resuspended in the desired medium or buffer for further analysis or experiments. Quality control and viability

assessment were performed by cell counting and viability assessment using a automated cell counter Nexcelom Cellometer Auto T4 (Nexcelom Bioscience, USA).

Cells were washed with PBS to remove any residual media or serum. The cell concentration ($5 \cdot 10^6$ cells/mL). The cells were then resuspended in 1X binding buffer provided with the Annexin V-FITC/PI staining kit (PharMingen, Becton Dickinson, USA). Annexin V-FITC was added to the cell suspension. The cells were incubated in the dark at room temperature (18°C) for a specified time (25 minutes) to allow Annexin V-FITC binding. After Annexin V-FITC incubation, propidium iodide (PI; PharMingen, Becton Dickinson, San Diego, CA, USA) was added to the cell suspension (5 µg/mL). The cells were incubated for an additional 5 minutes in the dark at room temperature (18°C) to allow PI staining. Following staining, the cells were analyzed using a flow cytometer equipped with appropriate filters for FITC (green) and PI (red) fluorescence. Compensation controls and gating strategies were set up to distinguish between live (Annexin V-FITC negative, PI negative), early apoptotic (Annexin V-FITC positive, PI negative), late apoptotic/necrotic (Annexin V-FITC positive, PI positive), and necrotic (Annexin V-FITC negative, PI positive) cell populations. Flow cytometry data was acquired and analyzed using software such as FlowJo or BD FACSDiva to quantify the percentages of different cell populations based on their Annexin V-FITC and PI staining patterns. The flow cytometry data was interpreted to determine the extent of apoptosis and necrosis in the cell population. Apoptotic indices, such as the ratio of early apoptotic cells to total cells (early + late apoptotic), were calculated to assess apoptosis induction.

Statistical analysis

The values in this investigation are presented as mean ±SD. Data were analyzed by one-way analysis of variance (MANOVA). Differences were considered statistically significant at a *P*-value of less than 0.05 (Statistica® 12.6, StatSoft, USA).

Results

The beginning of lactogenesis was characterized by a certain activation of the apoptotic process in the phagocytic defense system: the relative (14.54 ±0.52%) and absolute quantity ($103.3 \pm 7.57 \cdot 10^9$ cells/µL) of neutrophils with signs of apoptosis increased in the peripheral bloodstream (Table 1).

Table 1

Changes in cell apoptosis in peripheral blood of cows during different lactation periods

Lactation periods	The intensity of spontaneous apoptosis (ISA) [%]						CAB (L : M : N)
	lymphocytes		monocytes		neutrophils		
	%	· 10^9 cells/μL	%	· 10^9 cells/μL	%	· 10^9 cells/μL	
Colostrum period (n = 28)	3.72 ±0.46	26.37 ±1.45	1.8 ±0.40	7.73 ±0.34	14.5 ±0.52	103.3 ±7.57	0.23 ±0.06
Mid-lactation period (n = 28)	4.57 ±0.50*	35.4 ±1.71**	0.53 ±0.05**	4.12 ±0.45**	5.07 ±0.26**	39.3 ±1.52**	0.89 ±0.10
Drying off (n = 28)	5.16 ±0.30**	37.99 ±2.15**	0.79 ±0.04*	8.07 ±0.75*	12.08 ±0.40*	91.14 ±2.14*	0.43 ±0.03
Dry period (n = 28)	5.36 ±0.49**	36.88 ±2.35**	1.18 ±0.39	8.28 ±0.83**	15.27 ±0.45*	107.6 ±5.54*	0.31 ±0.07**

Explanations: * – $P < 0.05$; ** – $P < 0.01$ – regarding indicators at the beginning of lactation; L – lymphocytes; M – monocytes; N – neutrophils; CAB – Cell Apoptosis Balance

The apoptosis index of monocytes, in this case, was 1.8 ±0.40%, which is also associated with the direct involvement of these cells in the morphology and functional restructuring of the mammary gland parenchyma, as well as the active elimination of dead neutrophils. During the colostrum secretion period, the apoptosis index of lymphocytes was the lowest, amounting to 3.72 ±0.46%, which was also reflected in the lowest quantitative value (26.37 ±1.45 · 10^9 cells/μL). In the early lactation period, there was also a certain redistribution of individual populations of cells, which was reflected in the high value of the lymphocyte-to-monocyte-to-neutrophil (L : M : N) population ratio (0.23 ±0.06) and the monocyte-to-neutrophil (M : N) ratio (0.08), informative indicators of phagocytic cell apoptosis predominance (Figure 1). In the middle period of lactation, changes in the intensity of cell apoptosis primarily affected neutrophils in peripheral blood. As a result, the spontaneous apoptosis index of microphage cells decreased by 9.47% ($P < 0.01$), which was reflected in their absolute quantity (39.3 ±1.52 · 10^9 cells/μL), compared to the initial value of 103.3 ±7.57 · 10^9 cells/μL. Simultaneously, the intensity of spontaneous physiological death of monocytes decreased, while the spontaneous apoptosis of lymphocytes slightly increased and amounted to 4.57 ±0.50% (35.4 ±1.71 · 10^9 cells/μL). In the post-colostrum period, lymphocytes also undergo changes in their functional activity. The apoptosis of the investigated immune-competent cells remained within the range of physiological constants, as indicated by the changing parameters L : M : N (0.89 ±0.10) and M : N (0.10 ±0.01) ratios.

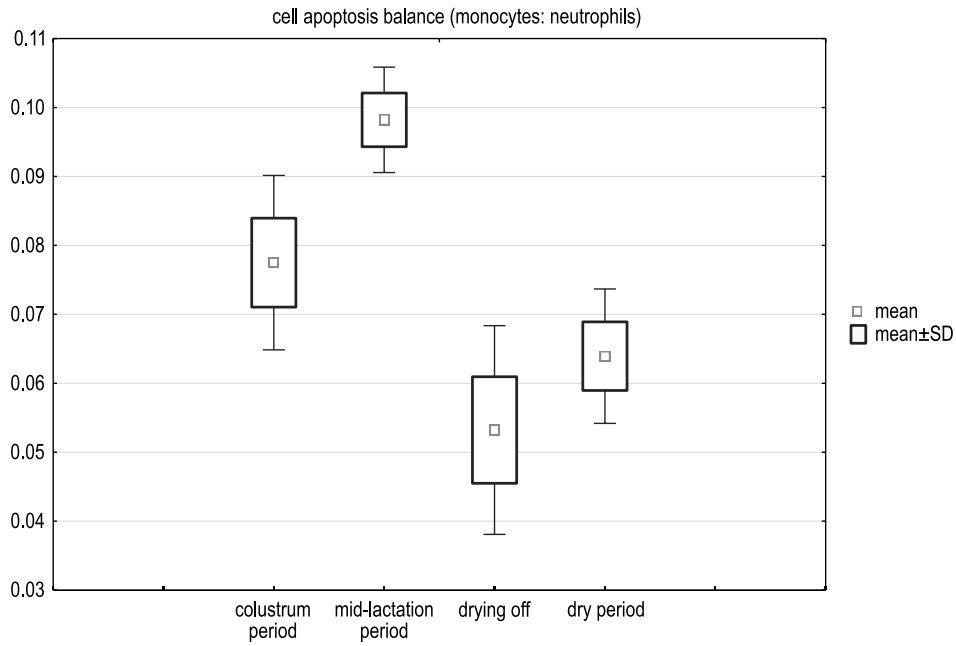

Fig. 1. The balance of apoptosis of phagocytes (Monocytes: Neutrophils) of cows in different periods of lactation

The initial involution processes in the mammary gland, which began during the drying-off period, continued into the dry period and were reflected in the process of spontaneous apoptosis of the investigated blood cells. The proportion of apoptotic neutrophil granulocytes observed during this time was at the level of 15.27 ±0.45% (107.6 ±5.54 · 10^9 cells/μL). There was also an increase in the proportion of monocytes that completed their life cycle (1.18 ±0.39%, 8.28 ±0.83 · 10^9 cells/μL). The population ratio in the L : M : N formula during the drying-off period was 0.43 ±0.03 and 0.31 ±0.07, indicating a cellular redistribution of immune competent cells towards the activation of spontaneous apoptosis in phagocytic cells, which directly participate in the elimination of metabolites expelled from the cow's mammary gland.

Discussion

Apoptosis, also known as programmed cell death, plays a crucial role in maintaining the balance and functionality of the immune system. This process is vital for various aspects of immune function, including the elimination of damaged or infected cells, regulation of immune responses, and

development of immune cells (PSAILA et al. 2016, LI et al. 2022). One key importance of apoptosis in the immune system is its role in eliminating cells that are no longer needed or have become harmful. For example, during an immune response, activated immune cells such as T-cells and B-cells undergo apoptosis after they have completed their tasks (LEVIN et al. 2016, KYDONAKI et al. 2021). This helps prevent excessive immune activation and inflammation, which can be detrimental to the body (HEISER et al. 2018, ZHELAVSKYI et al. 2021). Apoptosis is essential for removing self-reactive immune cells, which can cause autoimmune diseases if left unchecked (THEURL et al. 2016). Through apoptosis, immune cells that mistakenly target the body's own tissues are eliminated, contributing to immune tolerance and preventing autoimmune reactions. Apoptosis is involved in shaping the immune cell repertoire during development (SCHNABEL et al. 2020, SONG et al. 2022). Apoptosis plays a role in immune cell homeostasis, helping to maintain a balance between different immune cell populations (ZHELAVSKYI et al. 2023a). Excessive cell proliferation or survival can lead to immune disorders, and apoptosis helps regulate the numbers of immune cells to ensure optimal immune function (SUN et al. 2018, LIU 2020, ZHELAVSKYI et al. 2023b).

There is published data that various factors can affect the process of apoptosis of the blood cells of cows, including the inhibited level of the inflammatory factor in the peripheral circulation. In particular, there is information that apoptosis can be induced under the influence of excessive concentrated feeding of animals (ZHOU et al. 2024). In this study, we examined the mechanisms that maintain immune homeostasis during different stages of lactation. We confirmed that in different periods of lactation in the peripheral blood of cows, dynamic changes occur in the cell population with the manifestation of apoptosis. This discovery opens a new way to diagnose bovine mastitis and monitor the effectiveness of treatment and provides insight into how to perform effective immunomodulation.

During the early lactation period, a portion of neutrophil granulocytes, which had fulfilled their function, also underwent apoptosis, confirming our initial assertion made at the beginning of the experimental series. The issue of apoptosis in immune-competent cells of the mammary gland has attracted the attention of other researchers as well (ZHOU et al. 2015, BAE et al. 2022).

Bacterial components such as lipopolysaccharides can activate the immune defense of the mammary gland, leading to the recruitment of immune cells like neutrophils, macrophages, and lymphocytes to the site of inflammation. Mammary epithelial cells not only serve as a barrier but also respond to antigens by producing an inflammatory response. Intersti-

tial fibroblasts also play a role in the inflammatory process (XU et al. 2021). When the mammary gland faces bacterial invasion, macrophages in both breast tissue and milk recognize the invading pathogens, triggering an inflammatory response (SUN et al. 2018, LIU 2020). They also release pro-inflammatory substances to attract neutrophils to the area of inflammation within the mammary gland, aiding in bacterial resistance. Healthy mammary gland cells in breast tissue and milk primarily consist of breast epithelial cells and macrophages, whereas diseased tissues and their secretions predominantly contain transformed macrophages that become neutrophils (ANWER et al. 2016, ZHELAVSKYI et al. 2023a). One of the key functions of apoptosis in mastitis is its involvement in the removal of damaged or infected cells from the mammary gland. When bacteria or other pathogens penetrate the udder and cause inflammation, mammary gland cells may initiate apoptosis to prevent the spread of infection and tissue damage. During mastitis, the immune system is activated, but excessive inflammation can be harmful to the mammary tissues. Apoptosis helps limit this inflammatory response by reducing the number of activated immune cells and restoring balance in the udder. Some studies indicate that imbalanced apoptotic activity may be associated with the development of mastitis. If apoptotic removal of damaged cells is not effective, it can lead to worsened inflammation and infection (AKARAPHUTIPORN et al. 2021, KHAN et al. 2024). Therefore, understanding and controlling the apoptotic process in mastitis are crucial aspects for improving treatment and preventing this disease in cows.

Conclusion

The cow's lactation is characterized by dynamic changes in the manifestation of the apoptotic process of immune cells in peripheral blood. The highest intensity of apoptosis is observed in phagocytic cells (neutrophils and monocytes) during the colostrum secretion period and mammary gland involution. Lymphocyte apoptosis remains within the limits of physiological homeostasis throughout the study. Studying the physiological constants of blood cell apoptosis will serve as a basis for developing informative methods for diagnosing mastitis in cows and implementing effective treatment measures. The findings obtained from this research are valuable for practical veterinarians and from a public health perspective.

Accepted for print 4.04.2024

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POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 39(1): 15–29, Y. 2024

DOI: 10.31648/pjns.9688

INHERITANCE STUDIES OF GRAIN YIELD IN AFRICAN YAM BEAN (*SPHENOSTYLIS STENOCARPA* (HOCHST EX. A. RICH.) HARMS)

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Key words: additive gene effects, African yam bean, dominance gene effects, epistasis, generation mean analysis, inheritance.

Abstract

African yam bean (AYB) is a tropical underutilized leguminous crop serving as food for man and feeds for animals. Despite its nutritional values, its production is limited by low grain yield. Overcoming this menace through breeding programme requires adequate information on the inheritance pattern of the traits responsible for the low grain yield. Two divergent grain yield potential of AYB accessions collected from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, were crossed to generate F_1 progenies. The F_1 was advanced to F_2 progenies. Backcrosses to the two parents (BC_1 and BC_2) were generated. The six generations obtained were evaluated in three agroecological environments. Generation mean analysis and scaling tests for grain yield and some grain yield-related traits were estimated and subjected to factorial analysis of variance at $P < 0.01$. The results revealed that at least one of the scaling tests was significant in all the studied traits. The dominance gene effects were larger than additive gene effects in the studied traits. Complementary epistasis was observed in pod weight, number of seed per pod, weight of 100 seeds per pod and seed set percentage while duplicative epistasis was observed in number of locules per pod, number of peduncles per plant, number of pods per peduncles, number of pods per plant, pod weight per plant, seed weight per plant and grain yield. Environmental variances were less than genotypic variances in all studied traits. Pod weight, number of seed per pod, weight of 100 seeds per pod and seed set percentage while duplicative epistasis was observed in number of locules per pod, number of peduncles per plant, number of pods per peduncles, number of pods per plant, pod weight per plant, seed weight per plant and grain yield exhibited high broad sense and low narrow sense heritability. The results further showed that both additive and non-additive gene actions are important in inheriting these traits. The findings obtained in this study suggested that reciprocal recurrent selection should be adopted to obtained higher yield in AYB.

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Introduction

African yam bean (AYB) (*Sphenostylis stenocarpa* [Hochst. ex. A. Rich.] Harms) is a leguminous crop belonging to the family Fabaceae (OLUWOLE et al. 2021, JUDE et al. 2022). Among all the members of the genus *Sphenostylis*. AYB is the most popular and of higher economic value (OJUEDERIE et al. 2016). The crop is of tropical Africa origin (ADEWALE 2011) with a wide genetic diversity. It is a neglected, underutilized, orphan, and under-researched tropical African crop that is on its track to extinction due to the little attention the crop has received from the farmers, the farm produces processors, consumers, and the crop scientists (SHITTA et al. 2016, GBENGA-FABUSIWA 2020). The adaptability of the crop to a wide range of environmental and soil conditions makes it cheap and accessible, serving as a good source of plant protein in food and providing nutritional security (GEORGE et al. 2020, SUMANDIARSA et al. 2021). Figure 1 present the image of AYB plant, roots and seeds.

Fig. 1. Image of AYB: *a*; *b* – AYB plant; *c* – AYB pod; *d* – AYB roots; *e* – AYB seeds
Source: Genetic Resources Centre, International Institute of Tropical Agriculture, Ibadan, Nigeria, access: 25.04.2024

The AYB is cultivated for its highly nutritious edible seeds, leaves, and tubers (AFOLABI et al. 2019, OJUEDERIE et al. 2021). These edible parts of the crop are rich in nutrients that can be compared with other legumes (OLUWOLE et al. 2021). The protein content in the grains varies between 17 and 30 mg/g depending on the accession (GEORGE et al. 2020) which is more than those of pigeon peas, common pea, and chickpea (EKPO 2006, OLUWOLE et al. 2021). The grain and tuber of AYB also has high mineral and vitamin content (BAIYERI et al. 2018, AREMU et al. 2019, NAMANI et al. 2021).

The flour from AYB seeds is used to fortify food that is low in protein content and used as a supplement in livestock feed (NAMANI et al. 2017). The crop is commonly used in replenishing agricultural soil due to it potentiality of fixing nitrogen. Considering the great potential AYB exhibited, its utilization will not only ensure food security but will also sustain agricultural land.

Despite the numerous economic value importance of AYB, the average grain yield of the accessions in farmers' possession over time is low thereby limiting its production and improved varieties with high grain yield attributes (ADEWALE 2011). It becomes necessary to have detailed information of the mode of inheritance of the yield and non-yield trait(s) involves and the nature of gene actions in determining the efficiency of any breeding procedure that can help to attain a maximum genetic improvement in the crop (FOUAD 2020).

Moreover, determining the gene effects governing quantitative traits of the crop is essential. Various biometrical methods have been previously used to obtain information about the inheritance of quantitative traits in crops. For instance, generation mean analysis, a biometrical method can provide the required information on the relative importance of mean effects of the gene(s) i.e. additive effects, dominance deviations, and its effects as a result of non-allelic genetic interactions or epistatic gene effects while estimating genotypic values of the individuals, average genotypic values of families and generations at large (MATHER and JINKS 1982). In this study, generation mean analysis was used to elucidate the mode of inheritance of grain yield and grain yield-related traits in AYB. This was with a view to provide information on the genetic materials required for the improvement of the crop.

Materials and Methods

Source of genetic materials

The genetic materials used for this research were two AYB accessions (TSs 24 and TSs 67) collected from the Genetic Resource Centre (GRC) of the International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria with divergent grain yield potentials. TSs 24 is a high grain yielding while TSs 67 is a low grain yielding accession. Information on the initial evaluation trials conducted at IITA, Ibadan regarding the two accessions on days to 50% flowering were noted to determine the accurate planting time of each accession to ensure proper flowering synchronization to achieve success during hand hybridization.

Obtaining F_1, F_2 and backcross population

Seeds of the two divergent parental lines were planted at the Biological Garden of Elizade University, Ilara-Mokin, and Ondo State, Nigeria. Each of the two parental lines were crossed to generate F_1 progenies. Some seeds from the F_1's and the two parents were planted to advance to F_2 progenies and backcrosses were generated. Backcrosses to P_1 and P_2 were done using F_1 (F_1 was crossed with P_1 and P_2 respectively). Genetic studies were conducted on the six generations generated; P_1, P_2, F_1, F_2, backcross to P_1 (BC_1) and backcross to P_2 (BC_2).

Evaluation of the generation

The parental lines (P_1 and P_2) and F_1 representing the non-segregating population along with F_2, BC_1, and BC_2 representing segregating population were evaluated for grain yield and grain yield-related traits such as pod weight, number of seed per pod, weight of 100 seeds per pod, seed set percentage, number of locules per pod, number of peduncles per plant, number of pods per peduncles, number of pods per plant, pod weight per plant, seed weight per plant and grain yield. These were further used to determine their mode of inheritance. The evaluation was carried out in three agro ecological environments representing three different agro-ecological zones in Nigeria at the Biological Garden, Elizade University, Ilara-Mokin, Ondo State, Nigeria (representing rain forest agro-ecological zone); Ekiti State University Agricultural Teaching and Research Station, Ado-Ekiti, Ekiti State, Nigeria (representing derived savanna agro-ecological zone), and Oke-Ako Farm Settlement, Oke-Ekiti, Ekiti State, Nigeria (representing southern Guinea savannah agro-ecological zone). Seeds of the six generations generated were planted on the field, set out in a Randomized Complete Block Design (RCBD) with three replicates in each of the experimental sites. The number of plants evaluated at each experimental site for each generation were; Experimental site I and II (Rain forest agro-ecological zone and Derived savannah agro-ecological zone) consists of: 21 plants for the non-segregating population P_1 and P_2; 90 and 89 plants for the backcross populations of BCP_1 and BCP_2 respectively.

Experimental site III (Southern Guinea savannah agro-ecological zone) consists of: 21 plants for the non-segregating population P_1 and P_2; 30 plants for the non-segregating population of F_1; 90 plants for segregation population of F_2; 30 plants for the non-segregating population of F_1; 51 plants for segregation population of F_2; 51 plants for the backcross population of BCP_1 and BCP_2.

Pollination procedure

The following steps were involves in emasculation and pollination in AYB flower:

1. Appearance of purple stripe along the flower wing is a sign of flower maturity and the readiness of the flower bud to anthesize within a day.
2. Sterilized forcep was carefully used to remove the petals and the stamen (kneel, banner and wing). When removing the flower petals and stamen with the sterilized forcep, second hand should be used to hold the flower securely and gently to avoid flower dropping from the peduncle.
3. The flower filaments and anthers were gently removed.
4. At this stage, the flowers had been successfully emasculated. The emasculated flowers were set for pollination.
5. For the emasculated flowers that were not ready for hand pollinated immediately, they were covered with small sized pollen bags.
6. A matured flower from a pre-determined plant was removed from its peduncle, tease and use as pollen source or donor.
7. The dehisced anther was rubbed on the emasculated flower. This was done carefully to avoid any form of contamination.
8. The pollinated bags were used to cover the pollinated emasculated flowers immediately.
9. The pollinated flowers were properly labeled and tag for easy identification.
10. The bags were removed a day after pollination.
11. Formation of pods from the hand pollinated flower is a sign of successful pollination.

Agronomic Practices

The experimental plots in the three environments were well-ploughed, harrowed, and ridged mechanically. Planting was carried out in the fourth week of May 2022 for evaluation when the rain was steady. The seeds were sown solely at 1m x 1m spacing at a depth of 2–3 cm. Staking was done two weeks after planting and seedlings were trail to the stakes continually. The plots were kept weed-free throughout the study. No incidence of pest infestation or disease infection was recorded throughout the research.

Data collection

Data were collected on grain yield and some grain yield-related traits (Table 1) using AYB descriptors (ADEWALE and DUMET 2010).

Table 1

The list and description of the quantitative traits collected

S/N	Traits	Abbreviation	Measurement definitions	Unit
1	number of pods per peduncle	PodPed.$^{-1}$	the mean number of pods per peduncle of 10 randomly selected plants from the same plot	counting; visual assessment method
2	number of peduncles per plant	Ped.Pl. $^{-1}$	the mean number of peduncles from 10 randomly selected plant sample at harvest from the same plot	counting; visual assessment method
3	number of locules per pod	Loc.Pod^{-1}	this is the mean number of seed cavities in 10 randomly selected pods from the same plot	counting; visual assessment method
4	number of seeds per pod	Seedpod^{-1}	this is the mean number of seeds from 10 randomly selected pods in the same plot	counting; visual assessment method
5	pod weight	Pw	this is the average weight of 10 randomly selected pods at harvest from the same plot using digital weighing balance	[g]
6	number of pods per plant	PodPl.$^{-1}$	this is the mean number of pods from 10 randomly selected plants in the same plot at harvest	counting; visual assessment method
7	pod weight per plant	PwPl.$^{-1}$	this is the mean weight of total pods produced by 10 randomly selected plants from the same plot using digital weighing balance	[g]
8	seed weight per plant	SwPl.$^{-1}$	this is themean weight of seeds produce per plant of produced 10 randomly selected plant from the same plot using digital weighing balance	[g]
9	100-seed weight	100-SW	the weight of 100 randomly selected seeds taken from total seed yield obtained from the same plot using electronic weighing balance	[g]
10	seed Set Percentage	SSP	the mean ratio of seed number and loculi number/ pod multiplied by 100; measured on 10 randomly selected pods	[%]
11	grain yield per hectare	GY^{-1}	the weight of total dried seeds obtained from the same plot using weighing balance.	[kg ha^{-1}]

Statistical analyses

Data obtained were subjected to factorial analysis of variance (ANOVA) using IRRI PBTools software, version 1.4. (IRRI 2014) by the Biometrics and Breeding Informatics, PBGB Division, International Rice Research Institute, Los Baños, Laguna based generation mean analysis. Estimates of the four scaling tests (A, B, C, and D) and Generation mean analysis were determined according to the method suggested by HAYMAN (1958) and JINKS and JONES (1958). The significances of scaling test A and B, C and D indicate the presence of *J* (additive x dominance interaction), *l* (dominance x dominance interaction) and *i* (additive x additive interaction). The significance of one of the four scales for a trait reveals that epistatic digenic interaction that could contributed to the inheritance in the traits.

Biometrical methods

Most biometrical methods presumed the absence of epistasis in drawing inferences from genetic studies, whereas its presence cannot be underestimated (SHARMA 1988). Most of these biometrical methods failed to estimate gene interaction effects. However, generation mean analysis (GMA) as a biometrical method proposed by HAYMAN and MATHER (1955), and JINKS and JONES (1958) is capable of handling the limitations of other biometrical methods efficiently (SHARMA 1988). GMA provides the opportunity to detect the presence or absence of epistasis (non-allelic interaction) and determined the type of epistasis that is involved if present (SHARMA 1988).

Results

Estimate of scaling tests for grain yield and grain yield-related traits

Estimates of scaling tests for grain yield and some grain yield-related traits in AYB across the three environments were presented in Table 2. Scaling test A for the combined agro ecological environments shows that the traits are significantly different ($P < 0.01$) for the number of locules per pod, the number of peduncles per plant, the number of pods per peduncle, pod weight per plant, seed weight per plant, weight of 100 seeds and grain yield. Scaling test B indicated that four such as number of peduncles per plant, number of pods per plant, weight of 100 seeds, and grain yield were significantly different ($P < 0.01$). For scaling test C, the traits studied were not significant for the number of pods per plant, pod weight per plant, pod

weight, and seed weight per plant whereas other traits were significant. Moreover, for scaling test D, all the traits were significant for all the studied traits except for seed weight per plant and weight of 100 seeds.

Table 2

Estimates of scaling test for grain yield and other related agronomic traits in six generations of AYB across the three environments

Traits (units)	Scaling test			
	A	B	C	D
$LocPod^{-1}$	-6.4 ±0.53**	-0.23 ±0.59	-4.17 ±1.05**	-1.23 ±0.42**
$PedPl^{-1}$	-1.89 ±0.25**	-2.02 ±0.29**	1.71 ±0.5**	2.81 ±0.21**
$PodPed^{-1}$	-1.39 ±0.08**	0.11 ±0.09	-0.42 ±0.16**	-0.43 ±0.06**
$PodPl^{-1}$	-1.22 ±0.89	-10.01 ±1.21**	0.83 ±1.90	-6.03 ±0.75**
$PwPl^{-1}$ [g]	-30.74 ±4.38**	6.06 ±5.89	-5.14 ±9.33	-9.77 ±4.15**
PW [g]	0.07 ±0.12	0.21 ±0.21	-0.42 ±0.28	0.35 ±0.14*
$SeedPod^{-1}$	-0.02 ±0.40	-0.08 ±0.48	-4.52 ±0.84**	1.85 ±0.34**
SSP [%]	-0.03 ±0.42	-0.72 ±00.47	-4.53 ±0.83**	1.89 ±0.35**
$SwPl^{-1}$ [g]	-17.68 ±6.31**	5.38 ±5.43	-3.6 ±9.78	-4.35 ±3.67
100SW [g]	2.97 ±0.68**	4.19 ±0.95**	6.96 ±1.35**	0.1 ±0.48
GY [kg hac^{-1}]	-150.02 ±15.90**	-674.7 ±4.22**	1302.52 ±86.31**	238.81 ±43.92**

*, ** Significant at $P < 0.05$ and $P < 0.01$ level of probability respectively

Explanations: $locPod^{-1}$ – number of locules per pod; $PedPl^{-1}$ – number of peduncles per plant; $PodPed^{-1}$ – number of pods per peduncle; $PodPl^{-1}$ – number of pods per plant; $PwPl^{-1}$ – pod weight per plant; PW – pod weight; $SeedPod^{-1}$ – number of seeds per pod; SSP – seed set percentage; $SwPl^{-1}$ – seed weigh per plant; 100SW – 100-seed weight; GY – grain yield ha^{-1}.

Estimates of gene effects for grain yield and some grain yield-related traits in AYB

Table 3 shows the estimates of gene effects for grain yield and some grain yield-related traits across the three agro ecological environments. The mean and the additive gene effects were highly significant ($P < 0.01$) in all the studied traits. The result also revealed that the dominance gene effect was significant ($P < 0.01$) for the traits studied except for the number of seeds per pod, seed set percentage, and weight of 100 seeds. Additive x additive gene interaction was also significant for all the studied traits except for seed weight per plant and weight of 100 seeds. Additive x dominance interactions were not significant ($p > 0.05$) for the number of peduncles per plant, seed set percentage, seed weight per plant, and weight of 100 seeds. Furthermore, the dominance x dominance gene interaction was highly significant for all the studied traits except seed weight per plant.

Table 3

Estimates of gene effects for grain yield and other related agronomic traits in six generations of AYB across the three environments

Traits	Gene effects						
	m	*d*	*h*	*i*	*j*	*l*	epistasis type
LocPod^{-1}	22.74 ±0.18**	-0.17 ±0.21**	7.42 ±0.96**	2.46 ±0.84**	6.17 ±0.72**	-9.09 ±1.35**	D
PedPl^{-1}	9.71 ±0.08**	-1.09 ±0.12**	7.20 ±0.47**	5.62 ±0.43**	-0.13 ±0.33**	-9.53 ±0.70**	D
PodPed^{-1}	2.66 ±0.02**	0.05 ±0.03**	0.89 ±0.14**	0.86 ±0.12**	1.5 ±0.10	-2.14 ±0.21**	D
PodPl^{-1}	19.67 ±0.30**	-8.45 ±0.44**	13.20 ±1.62**	12.06 ±1.50**	-8.79 ±1.28**	-23.29 ±2.6**	D
PwPl^{-1} [g]	113.5 ±1.67**	-38.65 ±2.46**	46.37 ±8.62**	19.54 ±8.30*	36.8 ±6.40**	-44.22 ±13.58**	D
PW [g]	4.79 ±0.05**	-1.25 ±0.1**	2.07 ±0.30**	-0.7 ±0.29**	0.14 ±0.22**	0.98 ±0.48**	C
SeedPod^{-1}	17.85 ±0.15**	-3.51 ±0.17**	5.82 ±0.78	-3.7 ±0.69**	-0.78 ±0.57**	2.88 ±1.09**	C
SSP [%]	17.80 ±0.16**	3.47 ±0.18**	10.01 ±0.78	-3.78 ±0.70**	-0.7 ±0.58	3.04 ±1.11**	C
SwPl^{-1} [g]	94.79 ±1.45**	-35.83 ±2.25**	41.74 ±9.31**	8.7 ±7.35	23.06 ±7.53	-21 ±13.29**	D
100SW [g]	22.96 ±0.17**	-2.76 ±0.33**	8.9 ±1.09	-0.2 ±0.97	1.22 ±0.98	7.36 ±1.91**	C
GY [kg hac^{-1}]	1460.69 ±21.57**	-581.67 ±8.19**	592.10 ±87.85**	477.62 ±84.43**	524.5 ±16.33**	-347.28 ±92.33**	D

*, ** Significant at $P < 0.05$ and $P < 0.01$ level of probability respectively

Explanations: locPod^{-1} – number of locules per pod; PedPl^{-1} – number of peduncles per plant; PodPed^{-1} – number of pods per peduncle; PodPl^{-1} – number of pods per plant; PwPl^{-1} – pod weight per plant; PW– pod weight; SeedPod^{-1} – number of seeds per pod; SSP – seed set percentage; SwPl^{-1} – seed weigh per plant; 100SW – 100-seed weight; GY – grain yield/ha^{-1}; *m* – mid point; *d* – additive effect; *h* – dominance effect; *i* – additive X additive gene interaction; *j* – additive X dominance gene interaction; *l* – dominance X dominance gene Interaction; D – duplicate type of epistasis; C – complementary type of epistasis

Estimates of components of variance and other allied genetic parameters for grain yield and grain yield-related traits in AYB

Estimates of components of variance and other allied genetic parameters for grain yield and grain yield-related traits in AYB across the three agro ecological environments are shown in Table 4. The values observed due to environmental variance were less than values obtained from additive and dominance variances in grain yield attributes. Environmental, additive, and dominance variances had the same value (0.01) for the number of pods per peduncle. The weight of 100 seeds were 0.24, 0.16, and 1.17 g for environmental, additive, and dominance genetic variances, respectively. This indicated that the value for environmental variances is greater than additive variance but less than dominance variance for this trait. A similar trend was observed in all the studied traits except for the number of pods per peduncle and grain yield. Pod weight recorded the same value (0.01) for environmental and additive variances. The result indicated that dominance genetic variance was higher than the additive genetic variance in all the studied traits.

Table 4

Genetic parameters for grain yield and other related agronomic traits in six generations of AYB across the three environments

Traits	σ^2E	σ^2D	σ^2H	DD	σ^2P	σ^2G	Hns [%]	Hbs [%]
100SW [g]	0.24	0.16	1.17	2.64	1.58	1.34	10.68	84.83
$LocPod^{-1}$	0.13	0.02	0.45	4.39	0.61	0.48	3.84	77.97
$PedPl^{-1}$	0.02	0.01	0.08	2.53	0.12	0.10	10.97	81.42
$PodPed^{-1}$	0.01	0.01	0.01	2.89	0.01	0.01	8.68	81.54
$PodPl^{-1}$	0.39	0.20	1.61	2.78	2.22	1.82	9.40	8229
$PwPl^{-1}$ [g]	7.72	6.57	32.90	2.23	47.20	39.47	13.92	82.62
PW [g]	0.01	0.01	0.03	1.62	0.05	0.05	24.90	90.59
$SeedPod^{-1}$	0.08	0.01	0.26	4.11	0.35	0.27	4.32	77.57
SSP [%]	0.09	0.02	0.27	3.55	0.37	0.29	5.77	78.69
$SwPl^{-1}$ [g]	14.28	5.91	60.52	3.19	80.72	66.43	14.80	82.29
GY [kg hac^{-1}]	0.54	796.56	1592.05	1.13	2389.16	2388.62	0.33	99.97

Explanations: $locPod^{-1}$ – number of locules per pod; $PedPl^{-1}$ – number of peduncles per plant; $PodPed^{-1}$ – number of pods per peduncle; $PodPl^{-1}$ – number of pods per plant; $PwPl^{-1}$ – pod weight per plant; PW – Pod weight; $seedPod^{-1}$ – number of seeds per pod; SSP– seed set percentage; $SwPl^{-1}$ – seed weigh per plant; 100SW – 100-seed weight; GY – grain yield/ha^{-1}; σ^2E – environmental variance; σ^2D – additive variance; σ^2H – dominance variance; DD – degree of dominance; σ^2P – phenotypic variance; σ^2G – genotypic variance; Hns [%] – narrow sense heritability in percentage; Hbs [%] – broad sense heritability in percentage.

Discussion

The estimate of the degree of dominance shows that none of the studied trait's values is less than 1. Grain yield recorded the highest value for phenotypic and genotypic variances. Furthermore, the variation observed for narrow sense heritability ranged from 3.84% for the number of locules per pod to 33.00% for grain yield. A range of 77.57 (number of locules per pod) to 90.59% (pod weight) was observed for broad-sense heritability.

The levels of significance observed in A, B, C, and D scaling tests show the presence of non-allelic interactions in the studied traits (SHARMA 1988). This reveals that epistatic digenic interaction contributed to the inheritance of all the studied traits. The scaling test results revealed that the additive-dominance model is insufficient for describing the mode of inheritance for grain yield and other studied traits in AYB. Most of the traits of interest to plant breeders show polygenic inheritance (ACQAACH 2012). This implied that the inheritance of these studied traits is complex and polygenic in nature (SHAHROLIN et al. 2013). This finding suggests that epistasis should be considered when proposing a successful improvement programme for all these traits in AYB. This finding is in conformation with the result of GUPTA et al (2017) for different traits in cowpea and grain yield in soybean by ABOU-SEN (2020).

The estimated mean (*m*) reveals the contribution owing to the overall mean coupled with locus effects and interaction of fixed loci. The level of significance in mean (*m*) values for grain yield and other studied traits across the three agro ecological environments is an indication that the traits were inherited quantitatively. JAGTAP (1986) reported that whenever the additive effects are more than the non-additive effects, it is suggested that selection would be effective in early segregation generation. On the other hand, if the non-additive effect is more than the additive portion, improvement of the traits involves selection through later generations. The results in this study revealed that dominance gene effects were larger than additive gene effects in some traits including grain yield in some environment(s) while additive gene effects were higher for some traits as well. This is an indication that both dominance and additive genes are important in the inheritance of these studied traits. Moreover, the estimates of dominance [*h*] effects that were significant for grain yield and in more than 90% of the studied traits across the three agroecological environments showed the importance of dominance gene action. The significance of both additive [*d*] and dominance [*h*] gene action in the inheritance of grain yield and some other studied traits across the three agroecological environments is an indication that both additive and dominance effects are essential in the genetics of these traits.

The type of epistasis in existence is determined by the dominance [*h*] effect and dominance x dominance [*l*] gene interaction effects. When the dominance [*h*] effect and dominance x dominance [*l*] gene interaction effects were in the opposite direction, a duplicate type of epistasis is said to be in existence. If the dominance [*h*] effect and dominance x dominance gene interaction effects were in the same direction, a complementary type of epistasis is involved (SHAMA 1988, SAID 2014, MISTRY et al. 2016). The different types of epistasis observed in grain yield and other studied traits could be because of environmental factors such as temperature, soil factors, relative humidity, water and wind. Complementary epistasis type was observed in pod weight, number of seeds per pod, seed set percentage, and weight of 100 seeds while other studied traits displayed duplicative epistasis. A duplicative type of epistasis hinders trait improvement through selection, thus high magnitude of dominance [*h*] and dominance x dominance [l] gene interaction effects would not be expected (MISTRY et al. 2016). Bi-parental mating is suggested for duplicate types of epistasis. Likewise in the presence of complementary type epistasis, improvement can be made through selection in F_3 onward (THAKARE et al. 2017).

There is preponderance of dominance variances in all the studied traits across the three agro ecological environments. Therefore, breeding for these traits should be based on hybridization (SAID 2014). The environmental variance values were lower than genotypic variance values in all the studied traits. This suggests that the inheritance of these traits is more determined by genetic factors rather than environmental factors, hence selection would be the best breeding approach to improve these studied traits (BARAKAT 1996, ALPHONSU et al. 2011, OYIGA and UGURU 2011). The number of pods per peduncle had the least environmental variance while seed weight per plant had the highest environmental variance although still lower compare to genetic variances. This suggests that environmental factors have a greater influence on seed weight per plant than every other studied trait. This shows that post-flowering environmental factors might influence grain filling.

When the degree of dominance equal to 0, it indicated that dominant gene action is totally absence. When the degree of dominance is equal to unity (1), this shows that complete dominance genetic action is in control. However, if it is greater than 0 but lesser than 1, it shows that partial dominance action is in existence. Moreover, whenever the degree of dominance is greater than 1, over-dominant genetic action is in existence (ELIA et al 1997, AGBOWURO 2016). In this study, the degree of dominance values was greater than unity (1) in all the studied traits in the combined environment. This shows the presence of over-dominance gene action in the inher-

itance of the studied traits. Therefore, early selection of these traits must be avoided. The selection should be delayed to the third or fourth generation to give room to the loss of non-additive genetic variance. After a series of inbreeding, the additive genetic variance can be estimated (SAID 2014).

The heritability percentage obtained for all the study traits in this study is greater than 60%. This indicated that reasonable progress can be made in improving these traits through selection. Similar findings were reported in Cowpea by SAID (2014). Narrow sense heritability in percentage was low for all the studied traits ranged from 0.33 for grain yield to 24.90 for pod weight according to ROBINSON et al. (1955) classification. Low narrow sense heritability in the study could be because of large epistatic effects. This was also buttressed by the findings of HAKIZINMANA et al. (2004). The differences observed between narrow and broad sense heritability reveals the contribution of non-additive effects (dominance and/ or epistasis) in the genetic makeup of the studied traits. Heritability for self-pollinated crops l is not important as narrow-sense heritability which is measured by additive variance directly. The differences recorded between the genotypes for the studied traits show that selection could be effective for making improvements in grain yield. This therefore implied that both additive and non-additive genetic actions are vital for improving grain yield in AYB.

Conclusion

The level of significance observed in scaling tests A, B, C, and D for grain yield and related grain yield traits studied in AYB reveals the presence of epistasis. This shows that epistasis should be considered when proposing a successful improvement programme for all these traits in AYB. The significance of both additive [*d*] and dominance [*h*] in the inheritance of the studied traits is an indication that both additive and dominance effects are essential in the genetics of these traits. The existence of the duplicative type of epistasis was exposed in some traits while complementary epistasis type was revealed in some other traits as well. Broad sense heritability values are higher is greater than narrow sense heritability in all the studied traits. Low narrow sense heritability values recorded for the studied traits are an indication that the non-additive gene is in control while high broad sense heritability shows that these traits are governed by additive genes with large heritable variance. Hence, recurrent reciprocal selection method should be adopted.

Accepted for print 15.05.2024

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POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 39(1): 31–52, Y. 2024

DOI: 10.31648/pjns.9762

RARE SPECIES OF BIRDS AND THEIR BIOTOPE DISTRIBUTION IN THE UNREGULATED PART OF THE LOWER TRANSBOUNDARY RIVER DESNA (KYIV, UKRAINE)

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Key words: rare species, birds, Desna River, biotopes, Bern conventions.

Abstract

This study examines avian diversity on a 15,000 hectare section of the 3,382,000 hectare Desna River floodplains over four years, revealing 48 species with significant conservation status across 24 identified biotopes, including 14, biotopes listed under Resolution 4 of the Bern Convention. Of these species, 40 are included under Resolution 6 of the Bern Convention, and 19 are listed in the Red Book of Ukraine. Additionally, there are 22 nesting species, 9 of which are in the Red Book of Ukraine, and 8 are recognized by the IUCN and the European Red List as near-threatened and vulnerable. The findings highlight the critical need for biotopes conservation and propose the establishment of "Podesinnia" National Park as part of a broader ecological strategy aligned with the NATURA 2000 objectives, underscoring the importance of international collaboration in biodiversity preservation efforts.

Introduction

The war has been ongoing in Ukraine for two years now. In the initial months of the full-scale invasion by the Russian Federation, hostilities took place around Kyiv and affected the only unregulated river in Ukraine, the Desna. To complicate the advancement of the occupying army towards the capital, bridges over this river were blown up, but the Russian army deployed pontoon crossings and forced the river. After the Russian army withdrew from the outskirts of Kyiv in the summer of 2022, the Desna riverbed was cleared of sunken military equipment. In the river's floodplain, sappers are still finding remnants of aviation munitions and artil-

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lery shells that did not explode. Moreover, the airspace over this floodplain continues to be used during mass missile strikes on Ukraine's capital. Therefore, there are currently no opportunities to conduct research in this area to update data on biodiversity.

The Desna River, the longest tributary of the Dnipro River, is distinguished by its swift current and marshy relief of adjacent territories, making it a unique hydrological object within Ukraine, unaffected by Soviet industrialization. In contrast, most Ukrainian rivers have undergone flow regulation, loss of forest masses in floodplains, marsh drainage, and intensification of agricultural use of meadow lands. As a result, the 591 km of the Desna River and 3,382,000 hectares of its valley and floodplains remain important for ecosystem conservation within the "Eastern Plains" ecoregion. The untouched natural mosaic of landscapes in the floodplain territories of this transboundary water artery supports populations of both locally and globally rare fauna species, especially birds. The Desna River, unlike many other tributaries of the Dnipro located in the Kyiv region, features optimal conditions for the existence and reproduction of various ecological groups of macrobenthos and ichthyofauna (SYTNIK 2012), which are part of the food chain impacting the number of nesting pairs of waterfowl (VASYLYUK et al. 2010). The ecological corridor along this river spans a considerable length and includes various biotopes (DUBROVSKY et al. 2008), which serve as sites for forming a number of ecological niches (FULLER, 2019). Such areas, being "hot spots," play a leading role in the conservation of global biodiversity (HU 2015, VAN DEN BERGH 2000, CANTONATI 2020). The identification of similar refugia in Ukraine is important in the context of expanding the NATURA ecological network (VASYLYUK et al. 2019). It is worth noting that the modern loss of agricultural lands, especially in the south of Ukraine, poses a risk of transforming natural meadow ecosystems in the Kyiv region into agricultural fields. This prospect raises concerns among ecologists and scientists, as the floodplain area of the Desna River serves as a biotopes for a number of nesting bird species, particularly those rare in Ukraine and the European Union. In light of the above, there is a current need to publish the results of monitoring studies in international scientific journals to highlight the ecological significance of the lower course of the Desna River. Additionally, this information may prove useful in assessing ecological losses and contribute to the development of strategies for restoring natural biotopes affected by military actions caused by the Russian Federation.

Methodology

From the 3,382,000 hectares of the unregulated valley of the Desna River, we have studied 15,000 hectares along the floodplain. Various biotopes within this area were subject to study. The classification of biotopes was carried out according to the categories listed in CHYTRY et al. (2020), DAVIES et al. (2004), ONYSHCHENKO (2016).

The study of the ornithofauna was conducted using the point-count method from 2018 to 2021. For travel between counting points, we used a car, and local movements were made on foot. At selected points, durations of stay ranged from 20 minutes to 1 hour (HUTTO et al. 1986, BIBBY 1998). The greatest focus was on the nesting period (observations were conducted once a week from the second week of April to the second week of July). During other periods (March, the first half of April, August, September, October, November), observations were done biweekly. Data from winter period observations (December-February) were also considered. The status of the birds was characterized according to the methodology presented by FESENKO and BOKOTEY (2002, 2007). The research subjects were rare bird species listed in the Red Book of Ukraine, IUCN, and the European Red List, as well as species included in the lists requiring conservation and special measures for the preservation of their biotopes, including migratory species – according to Resolution 6 of the Bern Convention (1996) (Convention on the Conservation… 2024, Revised Annex I of Resolution 6… 1998). The authenticity of nesting was determined using criteria recommended by the Committee of the European Ornithological Atlas – EOAC (Breeding Bird Atlas of Europe 1992). The taxonomy and nomenclature of birds in this article follow FESENKO (2022). This research is notable for its focus on recording species within their natural habitats for feeding and breeding, which is crucial for the conservation and restoration of populations in specific biotopes. Research stations and their coordinates are depicted in Table 1 and Figure 1–3.

Table 1

Geographical location of stations where research was conducted

№	Station	Latitude	Longitude
1	2	3	4
1	Desna river, Muromets Island	50.552217	30.544875
2	Desna river, Pogrebinska oxbow lakes	50.553579	30.598764
3	Desna river, v. Pogreby	50.572909	30.606280
4	Desna river, v. Zazimye	50.591977	30.659718
5	Desna river, v. Novosilky	50.611507	30.628081
6	Desna river, v. Pukhivka	50.613656	30.704848

cont. Table 1

1	2	3	4
7	Desna river, v. Nizhnya Dubechnya	50.642639	30.673422
8	Desna river, v. Verkhnya Dubechnya	50.728195	30.689576
9	Desna river, v.Voropaev	50.767244	30.689685
10	Desna river, Europe Island	50.827280	30.760660
11	Desna river, Europe Island	50.824312	30.750931
12	Desna river, v. Zhukin	50.798258	30.722507
13	Desna river, v. Rozhni	50.658432	30.712974
14	Desna river, Lyubichiv island	50.783733	30.719339

Explanations: numbers *1–14* – see Figure 1

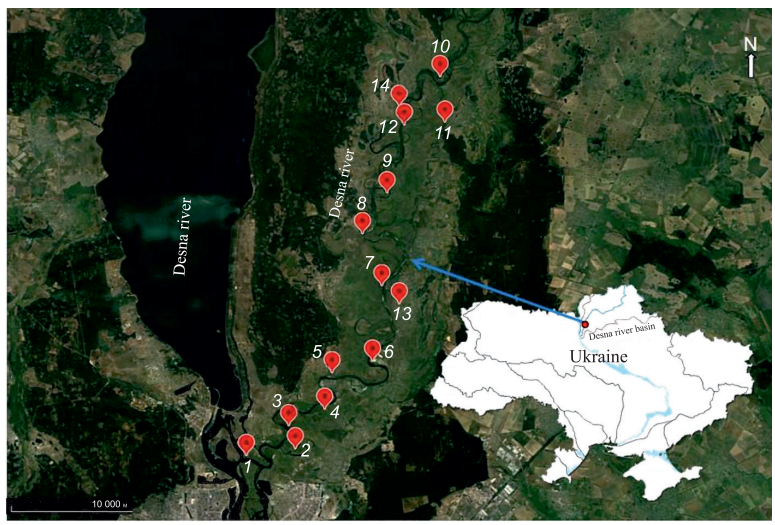


Fig. 1. Map of study areas

Explanations: numbers *1–14* – see Table 1

Source: Figure 1 is composed of a Google map and an Atlas of rivers of Ukraine (https://river.land.kiev.ua/atlas-rivers.html) as well as added author's elements (Yu. Kovalenko)

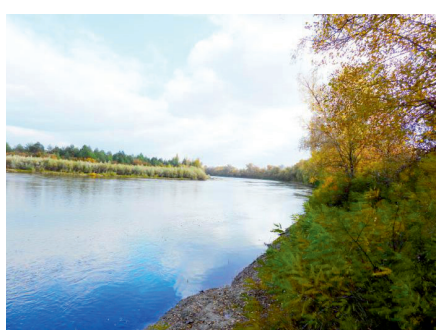


Fig 2. The Desna river (fragment a natural winding area near the village of Novosilka

Source: photo by M. Prychepa

Fig. 3. Swampy floodplain of the Desna river near the village Pogreby

Source: photo by M. Prychepa

Results

As a result of the biotope study in the Desna floodplain, 14 biotopes were identified that are listed under Resolution 4 of the Bern Convention. Additionally, there are 9 other biotopes currently not under protection (Table 2).

Table 2

Existing biotopes in the studied territory of the Desna River valley

Biotopes under Resolution 4	Other availables
C1.222. Hydrocharis morsus-ranae groupings (Floating Hydrocharis morsus-ranae rafts)	C3.1. Species-rich helophyte beds
C1.223. Stratiotes aloides groupings (Floating Stratiotes aloides rafts)	C3.2. Littoral groupings of tall helophytes (Water-fringing reedbeds and tall helophytes other than canes)
C1.225. Salvinia natans groupings (*Floating Salvinia natans mats*)	C3.6. Banks formed by soft and mobile deposits, with sparse vegetation or none (Unvegetated or sparsely vegetated shores with soft or mobile sediments)
C1.32. Free-floating vegetation of eutrophic waterbodies, groupings of the Lemnetea class in eutrophic conditions, includes vegetation dominated by the same species as the free-floating vegetation of mesotrophic waterbodies C1.22, but in eutrophic waterbodies	D5.1. High grass marshes (Reedbeds normally without free-standing water). Non-saline marsh groupings of *Phragmites australis, Phalaroides arundinacea, Scirpus lacustris, Typha* spp.
C1.67. Groupings at the bottom of dried-up waterbodies (Turlough and lake-bottom meadows). Groupings at the bottom of periodically, typically annually, dried-up standing waterbodies. At other times, this same area belongs to other settlements of groups C1 or C3	C3.6. Banks formed by soft and mobile deposits, with sparse vegetation or none (Unvegetated or sparsely vegetated shores with soft or mobile sediments)
C2.33. Vegetation of slow-flowing rivers with mesotrophic water	D5.1. High grass marshes (Reedbeds normally without free-standing water). Non-saline marsh groupings of *Phragmites australis, Phalaroides arundinacea, Scirpus lacustris*, *Typha* sp.
C2.34. Vegetation of slow-flowing rivers with eutrophic water	E1.D. Unmanaged xeric grasslands
C3.4. Species-poor beds of low-growing water-fringing or amphibious vegetation	E1.E. Trampled xeric grasslands with annuals
D5.2. Marshes dominated by large sedges (Beds of large sedges normally without free-standing water)	E2.7. Unmanaged mesic grasslands
E2.2. Low and medium altitude hay meadows	F9.2. Salix carr and fen scrub
E3.4. Moist and wet eutrophic and mesotrophic grasslands	–
F9.1. Riverine scrub	–
G1.11. Riverine Salix woodland	–
G1.3. Mediterranean riparian woodland	–

When these data are summarized according to the 2016 Biotope Catalog, six main biotope types emerge: continental water bodies (rivers, old riverbeds, lakes), meadows (dry and wet), shrubs, marshes, and floodplain forests (Figure 4–6). The distribution of these biotopes by area shows that moist meadows predominate in the studied territory, while the proportion of floodplain forests is represented by smler areas (Figure 7).

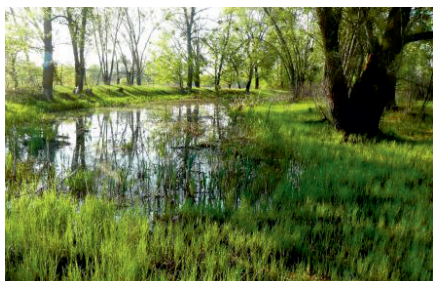


Fig 4. Floodplain forest on Myromets island
Source: photo by M. Prychepa

Fig 5. Swamps near v. Pogreby
Source: photo by M. Prychepa

Fig 6. Floodplain water body – old riverbed (fragment of Pogrebinska oxbow lakes.
Source: photo by M. Prychepa

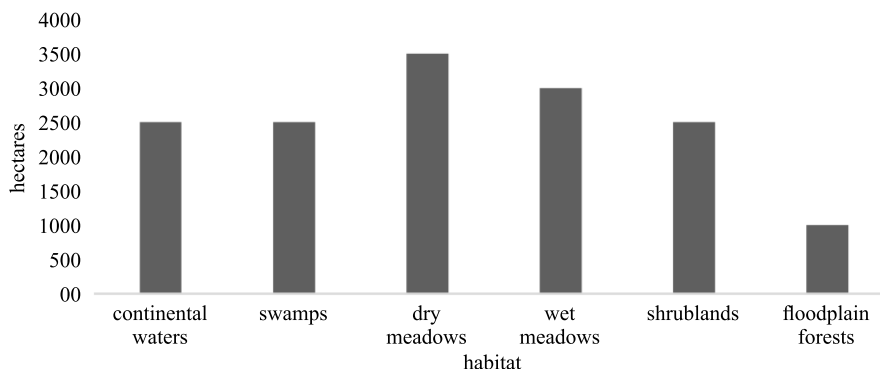


Fig 7. Distribution of areas in the studied part of the Desna River valley by types of biotopes, in hectares

The importance of this biotope distribution is highlighted in ornithological studies, which show the presence of 47 bird species belonging to 16 families, of which 3 species are included in the European Red List, 40 species are listed under Resolution 6 of the Bern Convention, and 20 species are included in the Red Book of Ukraine.

An analysis of the species richness of birds (Figure 8), according to the type of biotope, helps to understand the ecological interactions in the floodplain of the Desna River. It has been found that the largest number, 20 species, were registered in continental water bodies, which include rivers, old riverbeds, and lakes, and occupy approximately 15% of the studied area of the Desna River floodplain.

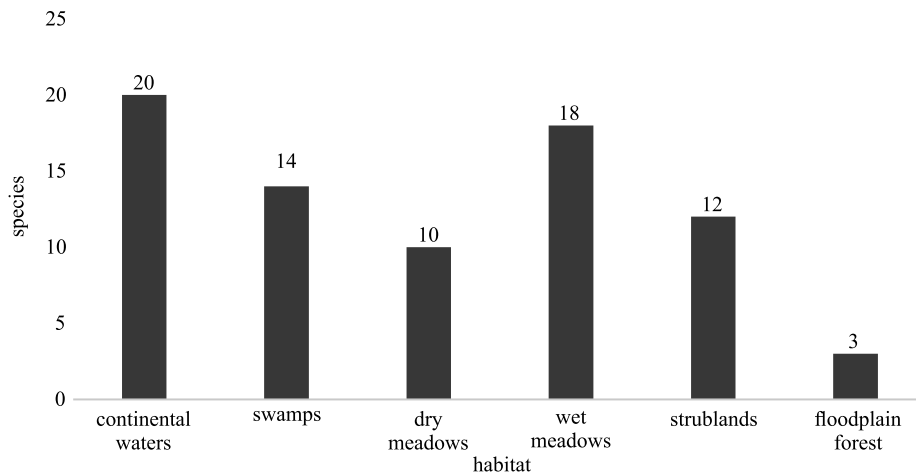


Fig. 8. Species richness of birds in the studied biotopes

In moist meadows, which cover 20% of the area, 17 bird species were found, including rare species such as Corn Crake *(Crex crex)*, which is also found in marshes and dry meadows. Additionally, these areas are regularly used for hunting by several birds of prey: Black Kite *(Milvus migrans)* (Figure 9), Short-toed Snake Eagle *(Circaetus gallicus)* (Figure 10), Western Marsh Harrier (*Circus aeruginosus),* Hen Harrier (*C. cyaneus).*

Marshes (sedge, reed, and bulrush) cover 15% of the biotope and host 14 bird species. This type of biotope is important for the conservation of nesting populations of Montagu's Harrier (*Circus pygargus*). Other rare species present include Purple Heron (*Ardea purpurea*), Eurasian Bittern (*Botaurus stellaris*), Little Bittern (*Ixobrychus minutus*), Eurasian Curlew (*Numenius arquata*), and European Honey Buzzard (*Pernis apivorus*).

Shrubs, occupying 15% of the territory, support the existence of 12 bird species and provide nesting for Great Grey Shrike (*Lanius excubitor*),

Lesser Grey Shrike (*Lanius minor*), Red-backed Shrike (*Lanius collurio*), European Nightjar (*Caprimulgus europaeus*), Wood Lark (*Lullula arborea*), and Barred Warbler (*Sylvia nisoria*).

Dry meadows cover 30% of the territory and are home to 9 bird species. This environment is used by species that prefer open spaces.

Floodplain forests occupy the smallest area (up to 5%). It is in this type of biotope that nesting populations of Black Kite *(Milvus migrans)* have been found.

Fig 9. Black Kite (*Milvus migrans*), representative of alluvial forest (included in the Red Book of Ukraine and Resolution 6 of the Bern Convention)
Source: photo by M. Prychepa

Fig. 10. Short-toed Snake Eagle
(*Circaetus gallicus*), representative of dry meadows and wet meadows (included in the Red Book of Ukraine and Resolution 6 of the Bern Convention)
Source: photo by M. Prychepa

The presence of various feeding grounds and trophic niches supports the presence of 48 bird species (Table 3) on the Desna River floodplain. The greatest species richness was observed during the spring-summer activity period, from May to July. In the winter period, Great Grey Shrike (*Lanius excubitor*) and Hen Harrier (*Circus cyaneus*) are present. Additionally, it is important to note the significant congregations of Common Goldeneye (*Bucephala clangula*) at the river's mouth, which is used by waterfowl during wintering.

Table 3

Bird species and their registration periods and biotope distribution in the floodplain areas of the lower Desna

№	Species	Registration periods	Biotopes					
			continental waters	swamps	dry meadows	wet meadows	shrubs-lands	alluvial forests
1	2	3	4	5	6	7	8	9
1	*Bucephala clangula* (Linnaeus, 1758)	October–March	+	–	–	–	–	–
2	*Mergus serrator* Linnaeus, 1758	October–November	+	–	–	–	–	–
3	*Caprimulgus europaeus* (Linnaeus, 1758)	May–July	–	–	–	–	+	–
4	*Crex crex* (Linnaeus, 1758)	May–July	–	+	–	+	–	–
5	*Zapornia parva* (Scopoli,1776)	May–June	–	+	–	+	–	–
6	*Grus grus* (Linnaeus, 1758)	March–April, October–November	–	+	–	+	–	–
7	*Haematopus ostralegus* (Linnaeus, 1758	April–September	+	–	–	–	–	–
8	*Vanellus vanellus* (Linnaeus, 1758)	March–September	–	–	–	+	–	–
9	*Numenius arquata* (Linnaeus, 1758)	September	–	–	–	+	–	–
10	*Limosa limosa* (Linnaeus, 1758)	April–August	–	+	–	+	–	–
11	*Calidris pugnax* (Linnaeus, 1758)	March–April, August–September	–	–	–	+	–	–
12	*Gallinago media* (Lathman, 1787)	May, September	–	+	–	+	–	–
13	*Xenus cinereus* (Guldenstadt, 1775)	August	+	–	–	–	–	–

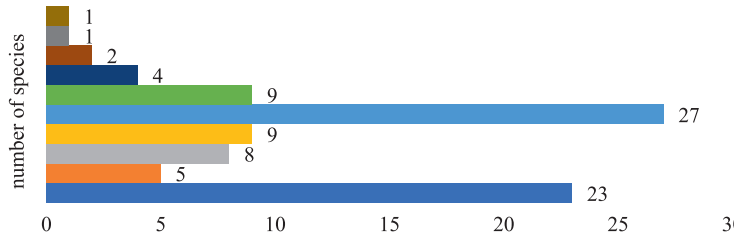
cont. Table 3

1	2	3	4	5	6	7	8	9
14	*Tringa glareola* (Linnaeus, 1758)	March–September	+	–	–	–	–	–
15	*Tringa totanus,* (Linnaeus, 1758)	April–August	–	–	+	+	–	–
16	*Sternula albifrons* (Pallas,1764)	May–July	+	–	–	–	–	–
17	*Sterna hirundo* (Linnaeus, 1758)	May–August	+	–	–	–	–	–
18	*Chlidonias hybrida* (Pallas,1811)	May	+	–	–	–	–	–
19	*Chlidonias leucopterus* (Temminck, 1815)	May–June	+	–	–	–	–	–
20	*Chlidonias niger* (Linnaeus, 1758)	May–July	+	–	–	–	–	–
21	*Gavia arctica* (Linnaeus, 1758)	October–November	+	–	–	–	–	–
22	*Ciconia nigra* (Linnaeus, 1758)	May–September	+	–	–	+	–	–
23	*Ciconia ciconia* (Linnaeus, 1758)	April	–	–	+	+	–	–
24	*Botaurus stellaris* (Linnaeus,1758)	April–May	+	+	–	–	–	–
25	*Ixobrychus minutus* (Linnaeus, 1766)	May–July	+	–	–	–	–	–
26	*Ardea purpurea* (Linnaeus, 1766)	June–August	+	+	–	–	–	–
27	*Ardea alba* (Linnaeus, 1758)	March–September	+	–	–	–	–	–
28	*Pandion haliaetus* (Linnaeus, 1758)	August–September, April	+	–	–	–	–	–
29	*Pernis apivorus* (Linnaeus, 1758)	June–September	–	–	–	–	+	+
30	*Circaetus gallicus* (Gmelin, JF, 1788)	April–September	–	–	+	+	–	–
31	*Clanga pomarina* (Brehm, CL, 1831)	April–September	–	+	+	+	+	+
32	*Clanga clanga* (Pallas, 1811)	March–October	–	+	+	–	–	–
33	*Hieraaetus pennatus* (Gmelin, JF, 1788)	July–August	–	–	+	+	+	–

cont. Table 3

1	2	3	4	5	6	7	8	9
34	*Aquila chrysaetos* (Linnaeus, 1758)	November	–	–	–	–	+	–
35	*Circus aeruginosus* (Linnaeus, 1758)	April–August	–	+	+	+	+	–
36	*Circus cyaneus* (Linnaeus, 1766)	March–April	–	–	+	+	–	–
37	*Circus pygargus* (Linnaeus, 1758)	May–September	–	+	+	+	–	–
38	*Milvus migrans* (Boddaert, 1783)	April–August	+	+	–	+	–	+
39	*Haliaeetus albicilla* (Linnaeus, 1758)	March–November	+	–	–	–	–	–
40	*Alcedo atthis* (Linnaeus, 1758)	April–November	+	–	–	–	–	–
41	*Falco vespertinus* (Linnaeus, 1766)	August–September	–	+	+	–	–	–
42	*Falco peregrinus* (Tunstall, 1771)	February–March	–	–	–	–	+	–
43	*Lanius collurio* (Linnaeus, 1758)	May–August	–	–	–	–	+	–
44	*Lanius minor* (Gmelin, JF, 1788)	May–August	–	–	–	–	+	–
45	*Lanius excubitor* (Linnaeus, 1758)	March–April	–	–	–	–	+	–
46	*Lullula arborea* (Linnaeus, 1758)	May–August	–	–	–	–	+	–
47	*Curruca curruca* (Linnaeus, 1758)	May–April	–	–	–	–	+	–
48	*Luscinia svecica* (Linnaeus, 1758)	April–August	–	+	–	–	–	–
Total			20	14	10	18	12	3

However, the graph (Figure 11) also illustrates the stark seasonal variations in species diversity, with the summer season supporting the greatest abundance of species. This influx likely reflects breeding activities and the availability of resources during this time. As the seasons transition from summer to autumn, there is a notable decrease in species count, which could be attributed to migration patterns and changes in the availability of trophic resources.



	1
spring – summer – – autumn – winter	1
autumn – winter – spring	1
spring – summer – autumn	2
winter	4
autumn	9
summer	27
spring	9
autumn – winter	8
summer – autumn	5
spring – summer	23

Fig. 11. Seasonal distribution of bird species on the desna river floodplain

In this context, the analysis of the ornithofauna by residency status allows for the determination of the species composition structure of birds in the floodplain of the river: nesting species constitute 58.3%, while migratory and wintering species account for 29.2% and 8.3% respectively (Figure 12).

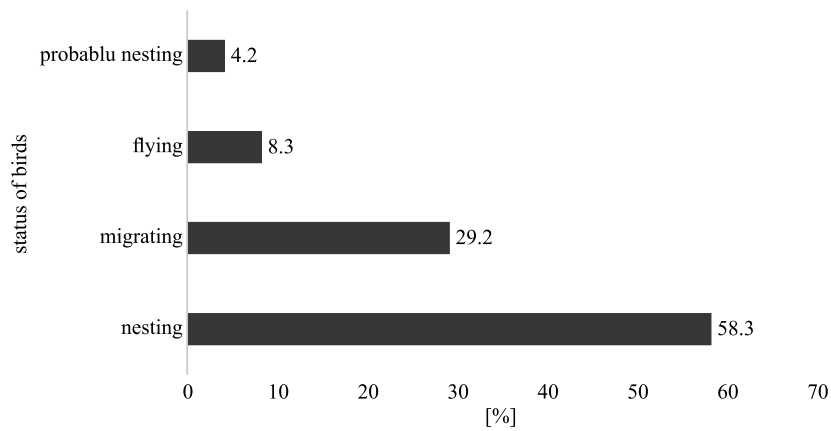


Fig. 12. Status of birds in the studied areas

Additional analysis, which shows the presence of birds from different ecological groups: limnophiles (55.2%), campophiles (20.0%), dendrophiles (23.0%), and petrophiles (2.1%), indicates favorable ecological conditions in the Desna River valley, particularly for the nesting of hydrophilic birds: Little Tern (*Sterna albifrons)* (10–40 pairs) and Eurasian Oystercatcher *(Haematopus ostralegus)* (3–4 pairs) – Table 4.

Table 4

Absolute numbers of individual representatives of the nesting fauna of rare birds (over a length of approximately 36.5 km)

№	Nesting	Number of pairs
1	Ciconia nigra	2–4
2	Milvus migrans	7–8
3	Circus pygargus	5–7
4	Circus aeruginosus	10–12
5	Circaetus gallicus	4–5
6	Aquilla pomarina	1–2
7	Haliaeetus albicilla	2
8	Crex crex	30–35
9	Limosa limosa	4–6
10	Vanellus vanellus	26
11	Tringa totanus	10
12	Pernis apivorus	2–4
13	Sterna albifrons	10–40
14	Sterna hirundo	4–8
15	Chlidonias leucopterus	4–8
16	Chlidonias niger	20–40
17	Haematopus ostralegus	3–4

The ecological value of the Desna Valley, reinforced by its comparison with biotopes included in Natura 2000, allows for considering the Desna River and its valley in a broader pan-European context (EU). Special attention is given to analyzing similarities between the floodplains of the Desna River and other rivers that already have conservation status in some neighboring and distant EU countries, as well as those sharing common features with the Desna River. Emphasis is placed on the similar area and number of bird species and biotopes that are protected in the European Union (Figure 13). The studied part of the Desna Valley in Ukraine covers 15,000 hectares and complies with the Birds Directive, as it provides a biotope for 42 species of birds listed under the Bern Conven-

tion and includes 14 protected biotopes. In neighboring countries, for instance, Ostoja Nidziańska in Poland covers 26,515.64 hectares with 26 bird species and 18 biotopes, while the Nida Valley includes 48 bird species over 19,956.08 hectares. The Danube meadows in Slovakia, spanning 16,511.58 hectares, contain 28 bird species and 12 biotopes. Pilis és Visegrádi-hegység in Hungary covers 30,145.74 hectares, protecting 41 bird species and 17 . In EU countries further from Ukraine, the German Vogelschutzgebiet "Unterer Niederrhein" covers 25,809 hectares and protects 60 bird species, the Dutch Rijntakken protects 37 bird species over 23,048 hectares, and the French River Seine nature reserve, the most diverse in terms of bird species, preserves 81 species over 18,592.61 hectares. All listed areas are protected under the Birds Directive, except for Ostoja Nidziańska (Poland) and Pilis és Visegrádi-hegység (Hungary) which are protected under the Directive.

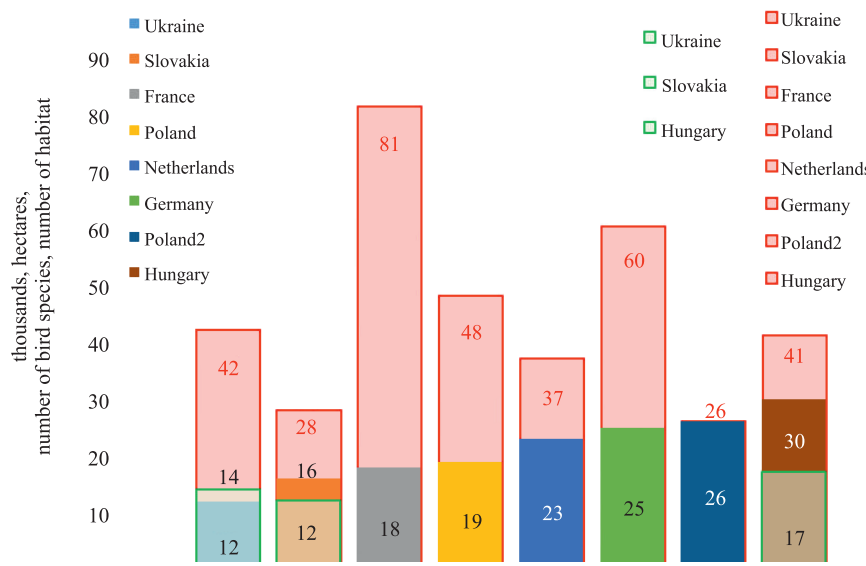


Fig. 13. The Desna floodplain compared to similar areas in neighboring and distant European Countries [in thousands of hectares in different countries]

Explanations: red represents bird species richness; green – the number of, and color-coded represents the area of protected territory

Discussion

Conducted by experts from the World Wildlife Fund (WWF), McGill University, and 30 international scientific institutions has shown that currently, out of the 246 longest rivers in the world, only 37% remain free-flow-

ing (GRILL et al. 2015, 2019, SCHAFER 2021) and only 10-30% of European floodplains remain in a natural state and have not lost their close connection with the river channel (TOCKNER et al. 2010, HEIN 2016, HERITAGE 2016, EEA 2019, TOCKNER et al. 2022). In Ukraine, such large rivers as the Dnipro, Siverskyi Donets, and Southern Bug, as well as their main tributaries, have been subjected to anthropogenic impact for a long time. From the 1930s to the 1980s, these rivers were regulated through the creation of hydraulic structures for intensive agricultural purposes (VISHNEVSKY 2000, KHILCHEVSKYI and GREBIN 2022). As a result, the ecological state of most rivers in the Dnipro basin was classified as "unsatisfactory" (YATSYK et al. 2007).

In the Kyiv region (central Ukraine), the expansion of agricultural lands leads to the plowing of meadows and the destruction of natural floodplain territories on the majority of small and medium-sized rivers. This has led to the formation of new agroecosystems, disrupting the typical biotopes of local campophilous birds: Tawny Pipit (*Anthus campestris*), Skylark (*Alauda arvensis*), Corn crake (*Crex crex*), Montagu's Harrier (*Circus pygargus*). It is worth noting that Montagu's Harrier – one of the most vulnerable species, the presence of nesting pairs of which indicates the conservation status of meadow-marsh biotopes, especially where there are associations of sedge (*Carex* sp.). A similar situation is observed in several EU countries. Currently, meadow biotopes both in Ukraine and in the European Union are becoming increasingly rare, leading to their protection (LISLEVAND et al. 2021). Thus, groups of dry and some types of moist meadows are protected under the Bern Convention and the EU Directive (Interpretive guide for settlements of Resolution No. 4 of the Berne Convention 2017, National biotope catalogue of Ukraine 2018). As floodplain landscapes are important for the conservation of wetland birds, the conservation level of the floodplain can be assessed by the number and species richness of birds (FERNANDEZ et al. 2005). In the Desna River valley, natural hydrology with typical floodplain complexes is preserved (VASYLYUK et al. 2010), which forms favorable conditions for nesting and reproduction of various bird species (AFANASYEV et al. 1992, DOMASHEVSKY 2005, GRISHCHENKO and YABLONOVSKA-GRISHCHENKO 2002, 2007, GRISHCHENKO et al. 1999, KOSTIUSHYN et al. 2020, MALYSHOK and KNYSH 2011, MOROZ 2015). Meanwhile, pan-European trends (BERG et al. 2002, MELTOFTE et al. 2018, LISLEVAND et al. 2021, ROODBERGEN and TEUNISSEN 2019), indicate a reduction in the population of nesting waders by approximately 40-50%, due to anthropogenic transformation of floodplain biotopes. In light of this, the International Union for Conservation of Nature has recognized the need to update the statuses for certain species

of waders from "LC" (Least Concern) to "NT" (Near Threatened) (BANIK 2016, SHYDLOVSKYY et al. 2017).

In response to the decline in populations of various bird species in Europe due to anthropogenic transformations in river basins, particularly the Seine and Danube, the EU has taken legislative steps to protect river valleys, including floodplain forests. In turn, the preserved diversity of landscapes and ecosystems in the Desna River valley supports the existence and reproduction of a large number of wetland birds, thereby ensuring genetic diversity. Regular spring floods further support these processes, preserving natural processes in the forest masses and meadows. Moreover, a reduction in agricultural impact in the vicinity of the studied area could further contribute to the restoration of natural plant groupings and increase the area for nesting of rare species (CHAMBERLAIN 2018). This is particularly relevant for meadow waders, whose population numbers depend on the groundwater level, which affects the height of meadow grasses in which they nest (RANNAP et al. 2016). At the same time, the decline in livestock farming across much of Ukraine affects the reduction of suitable environments for their reproduction (KOSTIUSHYN et al. 2020). Water level, water area, vegetation composition, and the size of wetland biotopes influence the biotope choice of wetland birds, particularly White-winged Tern (*Chlidonias leucopterus*), Black Tern (*Chlidonias niger*), Little Crake (*Porzana parva*), Great Snipe (*Gallinago media*). Bird species found in open biotopes where extensive agriculture is practiced, namely livestock farming (PUSTKOWIAK et al. 2021). However, intensive agriculture disrupts the integrity of the environment where the living space of Barred Warbler (*Sylvia nisoria)*, European Nightjar (*Caprimulgus europaeus)*, Red-backed Shrike (*Lanius collurio*) is concentrated (SALEK et al. 2022).

Measures have already been initiated to protect this area. Nature reserves such as the Dnipro-Desna Ornithological Reserve and the "Zhuravlyny" Ornithological Reserve have been established near the Desna River valley. Additionally, preparatory work is underway to create local reserves: "Ostrov Lyubychev", "Cherninsky", and "Urochyshe Rososchi". A crucial step for the conservation of wild flora and fauna along the Desna River is the creation of the "Podesinnya" National Park. This park will perform similar functions to other National Nature Parks in Ukraine. For example, the "Nizhnyosulsky" National Nature Park also plays a vital role in preserving natural floodplain areas: the mouth of the Sula River and part of the Dnipro water area. The "Pripyat-Stokhid" National Nature Park, which protects the riverbeds of the Pripyat and Stokhid rivers, has numerous branches that form labyrinths with marshy and sandy islands.

The "Kremenchug Plavni" Regional Landscape Park, located in the floodplain of the Middle Dnipro, includes the territory of the valuable Bilytskivskyi Plavni, preserving the natural appearance of the Dnipro floodplain as it was before the creation of the reservoir cascade. The park encompasses islands as well as the right and left bank floodplain areas.

Including significant floodplain areas around the city of Dnipro into the "Dniprovo-Orilsky" natural reserve exemplifies the importance of preserving river ecosystems in Ukraine. This helps to preserve key natural floodplains and islands on the left bank of the Dnipro. A similar strategy for protecting natural floodplain territories can be observed in Poland, where nature reserves such as "Cow Island" near Pulawy, "Vistula under Zawichost", and "Swiderskie Islands" in Warsaw, maintain the integrity of the natural floodplains of the Vistula River with its islands and spits. This underscores a pan-European approach to preserving river ecosystems, as in the case of the Lower Oder Valley located on the border between Poland and Germany, which is an example of river valley conservation in Central Europe.

Analyzing the importance of the Desna River floodplain for biodiversity conservation, particularly in terms of rare bird species, the need for protective measures becomes evident, especially during wartime. Although the Desna Valley is characterized by a high number of species with conservation status over a relatively small area, the lack of official conservation status exacerbates the threats to this natural heritage. Moreover, postwar population recovery requires significant efforts and often extends beyond a single country. For example, joint efforts by Germany, Portugal, and Gambia could contribute to the conservation of species whose populations are under threat, such as Limosa limosa (*Project LIFE19 IPE/DE/000004 Grass Bird* 2023). There are other examples of species conservation existing in the Desna River Valley. For instance, *Sterna albifrons* has become a conservation target in Croatia and other EU countries, where improvements to river biotopes, including sandy islands, help preserve the species (*Project Life14 NAT/HR/000115* 2024). Conservation efforts for *Ciconia nigra* in Croatia also include protecting wetlands important for the species' survival (*Project Life14 NAT/HR/000115* 2024). In Lower Saxony, restoration efforts are underway for meadows affected by drainage, intensification, plowing, and the removal of critically important environmental structures. Notably, this region is home to 19 males, approximately 20% of the entire *Crex crex* population. Over several years, the Lower Elbe has implemented a special conservation program that includes significant investments in land acquisition and improving meadow hydrology (*Project Life 97NAT-D-004233* 1997).

This comparison underscores the universality of efforts to protect natural floodplains and wetlands in the context of biodiversity and natural landscape conservation across different parts of Europe, which in turn demonstrates the need for broader international cooperation in conservation activities to preserve sensitive species and their breeding environments.

Conclusion

Investigation of avifaunal diversity conducted on a 15,000-hectare segment of the Desna River floodplains from 2018 to 2021 – an area representing a small fraction of the expansive 3,382,000 hectares – has unveiled a substantial assemblage of avian species. This survey revealed 48 species, of which 40 are listed in Resolution 6 of the Bern Convention, 7 are recognized on the European Red List, and 21 are inscribed in the Red Book of Ukraine, distributed across 6 studied biotopes. Within the lower Desna floodplain, there are a total of 24 biotopes, 14 of which are under the protection of Resolution 4 of the Bern Convention.

Meadow biotopes, both dry and moist, constitute the largest areas. Continental water bodies were found to be home to the highest species diversity, followed by wet meadows, while alluvial forests exhibited the lowest species variation. Seasonal observations indicated a peak in species richness during summer, with a notable decline towards autumn and a minimal presence in winter, highlighted by significant concentrations of the Common Goldeneye (*Bucephala clangula*). Among the nesting species, which represent a substantial 58.3% of the documented birds, three are registered with vulnerable categories on the European Red List: the Northern Lapwing (*Vanellus vanellus*) with 26 pairs, the Common Redshank (*Tringa totanus*) with 10 pairs, and the Black-tailed Godwit (*Limosa limosa*) with 4–6 pairs.

Given the Desna Valley's significant ecological value and resemblance to Natura 2000 biotopes, conservation measures are critical. The proposed "Podesinnia" National Park, alongside established and upcoming reserves, marks proactive steps for habitat protection. This initiative should be integrated into a wider strategy such as NATURA 2000. This research highlights the need for international cooperation in post-conflict restoration efforts, as seen in initiatives such as LIFE19 IPE/DE/000004 for habitat protection, and sets a precedent for future conservation strategies.

Acknowledgements

We are eternally grateful to Sergei Malomuzh for his participation in the expedition, as well as for his assistance in locating birds on the Desna floodplain.

Accepted for print 19.04.2024

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POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 39(1): 53–74, Y. 2024

DOI: 10.31648/pjns.9964

APPLICATION OF VIBRATIONAL SPECTROSCOPY FOR PLANT TISSUE ANALYSIS – CASE STUDY

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Key words: FT-Raman spectroscopy; specific leaf weight; spectra decomposition; phenolic compounds, carotenoids.

Abstract

Raman spectroscopy is a particularly advantageous method in plant biology, allowing simultaneous examination of various compounds and evaluation of molecular changes in plant tissues subjected to different stress factors. The purpose of our research was to investigate to what extent the differences in the physical properties of leaves of *Alnus viridis*, *Hieracium bifidum* and *Platycerium bifurcatum* allow us to reliably determine qualitative and quantitative changes in their chemical composition. We proved that if we employed the FT-Raman spectroscopy method direct comparison of the obtained results might be difficult or even impossible. Normalization of the spectra in some situations may help in the results interpretation. However, to study the global impact of the stress factors on the tissue we suggest preparing a tablet obtained from lyophilized and powdered leaves, that avoids the inhomogeneity of the sample. Additionally, the decomposition procedure of the overlapped peaks is necessary to obtain reliable quantitative results.

Introduction

There are a great deal of scientific reports concerning the analytical determination of pigments and biologically active substances such as phenolic compounds (BABARINDE and ADEOLA 2023, BOUYAHIA et al. 2022). One of the very convenient method of their analysis is Raman spectroscopy which is a very widespread technique in plant biology. Research includes

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algae, cyanobacteria and higher plants (GIERLINGER and SCHWANNINGER 2007, KULA et. al.2016, KULA et. al. 2014, PRATS-Mateu et. al. 2018, STAWOSKA et. al. 2021, ZEISE et al. 2018). In case of the latter ones, both seeds (LABANOWSKA et. al. 2016, SKOCZOWSKI et al. 2011, TROC et al. 2009), leaves and fruits (BOYACI et al. 2015, CHYLINSKA et. al. 2014, STAWOSKA et al. 2023) have been examined. This method has gained recognition, among others, due to the fact that it is non-invasive and does not require prior preparation of samples for the measurement. In the vast majority of cases, experiments are carried out directly on plant tissue. Raman spectroscopy has found a lot of applications – from simple measurements, allowing the determination of the occurrence of a particular type of compounds in a living organism (qualitative analysis), to a quantitative approach to the problem (PAYNE and KUROUSKI 2021, SCHULZ 2014). It has been proved that the discussed spectroscopic method allows the detection of substances responsible for the processes of photosynthesis (e.g. chlorophylls), photoprotection or mitigation of the effects of oxidative stress, especially carotenoids and phenolic compounds (SCHULZ and BARANSKA 2007). Raman spectroscopy is often used to look for and explain differences in the chemical composition of plant tissues, caused by various types of stresses (PAYNE and KUROUSKI 2021). Among others, the effects of abiotic stress, such as drought (ALTANGEREL et al. 2017, RYS et al. 2015), herbicides (SAJA et al. 2016) and wounding (LUKASZUK et al. 2017) are examined. Many experiments are also performed on the leaves of plants subjected to biotic stress (RYS et al. 2014). Furthermore, by performing the mathematical analysis (e.g. decomposition) of the spectra or their selected bands, it is possible to analyze e.g. the secondary structure of proteins in the tested samples (STAWOSKA et al.2020, STAWOSKA et al. 2009). Some scientists, however, consider the advantages of the technique overestimated and the results obtained in this way as not always reliable (DONG and ZHAO 2017). There are also other non-invasive and quick methods of direct analyses of photosynthetic pigments (CEROVIC et al. 2012). However, Raman spectroscopy is not suitable only for chlorophylls and flavonoids, but also for other biochemical components such as polysaccharides, fatty acids and proteins (CZAMARA et al. 2015, FARBER et al. 2019, SALETNIK et al. 2021, STAWOSKA et al. 2020, TALIK et al. 2020).

On the other hand, as with other experimental methods, this one also has its limitations. The fundamental phenomenon seems to be fluorescence. Some samples excited by visible radiation can induce electronic transitions, which in turn cause autofluorescence. In this way, the so-called fluorescent background makes the identification of Raman bands difficult or even impossible. The laser as a light source, introduced in the 1960s,

definitely contributed to the increased popularity of the method. Lasers emitting UV, Vis or NIR (with wavelengths up to 830 nm) light are usually used in dispersive Raman spectrometers. When lasers with wavelengths of 532 nm and 785 nm are used, the fluorescence signal is so strong that it often obscures the actual Raman signals of the substance. Therefore, a special solution was developed – a spectrometer with a laser with a wavelength of 1064 nm. Reducing the energy of laser radiation allowed the fluorescence signal to be reduced to such an extent that it was possible to identify substances whose spectra previously obtained with lasers were completely unreadable.

Although using the NIR excitation wavelength in FT-Raman is more suited compared to lower wavelength excitation, one can be still aware that autofluorescence is not eliminated at all and still remains a nuisance.

The next problem concerning the application of the FT-Raman spectroscopy method on a larger scale in plant research, however, is often the type of tissue used to measure. As mentioned earlier, the preparation of the material for analysis is not demanding. Thus, a quick analysis of the chemical composition may be easily performed. However, the natural environment plant growth conditions (e.g. light intensity and its spectral composition, various temperature condition, wind) strongly influence, among others, the structure and the physical properties of tissues (e.g. colour, leaf area and thickness). This, in turn, impacts the quality of the spectra obtained, and ultimately also the limitations (like lack of homogeneity of the tissues, possible problems with repeatability or changes in the presence of other compounds that may disturb the Raman spectra), should be taken into account during their analysis (Altangerel et al. 2017, Baranska et al. 2006, Bauer 2018, Dong and Zhao 2017, Krimmer et al. 2019). As we have observed, also the specific leaf weight (SLW) could be essential for the accuracy of the quantitative analysis. SLW is a physical parameter that affects the registered signal intensity. If plants of the same species grow in different thermal and light conditions in the field (under either shade or direct sunlight), they will have significantly different SLW. As a consequence, detected variations in spectral intensities are likely to be derived from different light absorption and scattering of tested leaves. It should be also pointed out that physical factors influencing the morphology of the leaves affect their chemical composition. Hence, the obtained results cannot be interpreted based on "raw data" and direct comparison of the obtained Raman spectra may lead to erroneous results. Solving this issue seems to be crucial also because scientists use the results obtained by Raman spectroscopy for chemometric analyses (cluster analysis) in which the intensity of the obtained signal is the most important (Rys et al. 2020).

The purpose of our work was to investigate to what extent the differences in the physical properties (namely SLW parameter) of leaves of three different plant species (*Alnus viridis*, *Hieracium bifidum*, *Platycerium bifurcatum*) allow to reliably determine not only qualitative but also quantitative changes in their chemical composition, using FT-Raman spectroscopy. In our research, we used leaves of plants belonging to different species, (a shrub, an herbaceous plant and a tropical fern) to propose an unambiguous and constant way of the preparation of the plant material, the spectra analysis and the interpretation of results.

Experimental

Materials and methods

Materials

The research was conducted for three plant species: *H. bifidum*, *A. viridis* and *P. bifurcatum*. To carry out the described experiments, leaves of the same plant species but with significantly different SLW parameter values were used. The differentiation of SLW values resulted from different plant growth conditions in the natural environment. Abiotic factors causing differences in the morphological structure of the leaves were strong sunlight, high temperature fluctuations of the day/night or differences in the intensity of light combined with the change of spectral composition. Naturally growing species, *H. bifidum* and *A. viridis*, were harvested from both shade or direct sunlight places. At least 10–20 leaves were collected from each growth place and 13 mm diameter roundels were cut out from them.

The nest leaves of *P. bifurcatum* (grew in a greenhouse of University of the National Education Commission, Kraków) were used as an experimental model possessing leaves with differential SLW parameter values (starting from the base to the apex of the leaf blade). The assay was performed for 2 morphologically different leaf parts. At least 4 leaves were collected from each growth places and again 13 mm diameter roundels were cut out from of them.

None of the tested plant specimens has been deposited in a publicly available herbarium. Detailed information is given below.

Alnus viridis. The plant specimens of *A. viridis* were obtained from a wild population located in the Bieszczady National Park. They were collected after obtaining permission from the park management (the topic of

the research no. 19/11, the license no. 48/12). Formal identification of *A. viridis* was done by Associate Professor Krzysztof Oklejewicz (Chair of the Botanic Department, University of Rzeszów). Green alder leaves – *A. viridis* came from positions located at various heights above sea level. The first harvest locality was at the top of Połonina Wetlińska (1215 m a.s.l.). The alder that existed here was a single shrub, growing in an open, well-lit area, on a ridge. The shrub was exposed to naturally changing weather conditions (strong sunlight and wind, large amplitude of daily temperatures) and was characterized by higher values of specific leaf weight (SLW – the method of determination is described below in the Methods section). The second locality was in post-agricultural areas, near the village Łobozew (568 m a.s.l.), in a location that was naturally a "protective umbrella" for the plant against strong sunlight, intense wind, or sudden rainfall. Shrubs in this locality were characterized by morphologically different leaves from those growing in Połonina Wetlińska and showed significantly lower values of SLW [42].

Hieracium bifidum. The leaves of *H. bifidum* were obtained from plants growing in their natural environment, in the Nature Reserve "Sokole Góry", in the Kraków – Częstochowa Upland. This is an area under partial protection, and the places where the research was carried out do not require any additional permission to conduct scientific investigations. Formal identification of *H. bifidum* was done by Professor Zbigniew Szeląg (ORCID ID: 0000-0002-7017-2823). Plants that grow in two different localizations were selected for experiments according to the conditions of full sunlight (plants from the southern wall of the rock, HL-plants, with higher value of SLW) and to the conditions of limited access of light (the shaded part, between the trees, LL-plants, with lower value of SLW).

Platycerium bifurcatum. The studies were performed using leaves of a six-year-old staghorn fern *(P. bifurcatum*). The plant specimens of *P. bifurcatum* came from the collection of the Institute of Biology and Earth Sciences, University of the National Education Commission, Kraków. They have been cultivated for years and are constantly available for research purposes. Formal identification of *P. bifurcatum* was done by Professor Andrzej Skoczowski (ORCID ID: 0000-0003-0334-9358). Plants were grown under a natural photoperiod in a greenhouse. Sporophyte has two types of leaves: the nest and the sporotrophophyll leaves. The nest leaves attach the plant to the trunk of the tree and collect minerals and water. Sporotrophophyll leaves are responsible for both assimilation and reproduction since they produce sporangia with spores. The Raman spectroscopic analyses were conducted using selected parts of blades of nest

leaves, namely the apex (with lower SLW values) and the base parts (with higher values of SLW).

Reagents and Solvents. Na_2CO_3 (p.p.a.), acetone (p.p.a.), methanol (p.p.a.), glacial acetic acid (p.p.a.) – Avantor Performance Materials Poland S.A., trans – ferulic acid, Folin and Ciocalteu's reagent – Sigma-Aldrich.

Methods. SLW Parameters Calculation

13 mm diameter roundels were cut out from the leaf blade of each of the tested plants (or its fragment – for *P. bifurcatum*), using a cork borer. Their area (a) was considered and then after the lyophilization, they were weighted, and their mass values (m) averaged. Based on the equation (1), the average density of the prepared roundels (Specific Leaf Weight) was calculated.

$$SLW = \frac{m}{a} \quad [g\ dm^{-2}] \tag{1}$$

where m means mass of the lyophilized roundels, a means area of the lyophilized roundels.

Preparation of tablets. The lyophilized leaves (160 mg) were grinded in a mortar. Next, the tablet was made using the equipment ABL&E-JASCO Polska Sp. Z o.o. (the diameter 13 mm, the pressure 200 atm). The prepared tablets were stocked in an exsiccator till the time of measurement.

Fourier transform raman spectroscopy and curve fitting. FT-Raman measurements were performed on dry leaves (or tablets obtained from lyophilized and powdered leaves of the same investigated plants) of *A. viridis*, *H. bifurcatum* and *P. bifurcatum* using a Nicolet NXR 9650 FT-Raman spectrometer equipped with a Nd:YAG^{3+} laser, emitting a beam at 1064 nm wavelength, and a germanium detector cooled by liquid nitrogen. The measurements were performed at room temperature, an aperture of 80 and a spectral resolution of 4 cm^{-1}. Spectra were recorded with a laser power of 0.4 W (it was monitored if it did not injure studied samples) and analyzed in the range 700–1800 cm^{-1}. Sixty-four scans were performed for each spectrum and each measurement was done in 4 to 8 repeats. The analysis of spectra was carried out using Omnic 8 and OriginPro 2017.

To determine the real contents of carotenoids and phenolic compounds, the band localized at 1480–1670 cm^{-1} was decomposed using PeakFit 4.12. The analysis started with a baseline correction that used a linear function.

In the next step, a second derivative of each measured spectrum was obtained, in order to find the number of components that built the band as well as their localization. Finally, a mathematical algorithm, employing Gaussian and Lorentzian functions was used, to iteratively estimate parameters using the method of least squares. In this method, the areas of selected peaks correspond to chemical vibrations that correlate with specified types of chemical moieties. For each obtained decomposition, the correlation coefficient was higher than 0.9986.

The biochemical assays of the total content of carotenoids, chlorophylls and phenolic compounds. The total content of carotenoids (and chlorophylls) in *P. bifurcatum* and *H. bifidum* was estimated according to the previously described (LICHTENTHALER 1987) and slightly modified procedure. In order to assess the quantity of these pigments, the extraction procedure was performed with the mixture of acetone: water (v/v: 98/2). The lyophilized plant sample (20 mg) was grinded in a mortar and the solution (1 mL) was added. The extraction was supported by ultrasonication for a higher yield (6 min, in an ice-water bath). In the next step the supernatant was collected, centrifuged (10 min, 3000 × g), and then decanted. The absorption spectra of the extract were recorded (3 repetitions) in the same solution, acetone: water (v/v: 98/2), in 1 cm path length quartz cuvette, in the spectral region 350–900 nm, with 0.2 nm precision, using spectrophotometer UV-VIS Analytic Jena, Germany. The absorption values were read at 470 nm, 644.8 nm and 661.6 nm and on the basis of their values, the chemical contents of chlorophyll *a*, C_a, chlorophyll *b*, C_b, and carotenoids, C_x, were estimated, according to the equations (2–4):

$$C_a = 11.24 \cdot A_{661.6} - 2.04 \cdot A_{644.8}, \quad (2)$$

$$C_b = 20.13 \cdot A_{644.8} - 4.19 \cdot A_{661.6}, \quad (3)$$

$$C_{x+c} = (1000 \cdot A_{470} - 1.90 \cdot C_a - 63.14 \cdot C_b)/214. \quad (4)$$

The chlorophylls contents, C*c*, were estimated only in order to calculate the total carotenoids content according to the equations given above. Therefore, the changes in the chlorophylls contents were not discussed in this manuscript.

All the preparation and measurements steps were done under the dim light because of the extremely high light sensitivity of the pigments. The quantitative assay was carried out immediately after the preparation of the extract.

The total content of phenols was estimated via the spectrophotometric method as well, using the Folin – Ciocalteu reagent. In the first step, the liquid extraction supported with ultrasonication was conducted. The lyophilized material (20 mg) was grinded in a mortar and then treated with

1 mL of the solution glacial acetic acid: methanol (v/v: 1/99). The mixture was closed under the nitrogen and then it was ultrasonicated within 6 min in an ice-water bath. Then the supernatant was decanted and centrifuged (10 min, 3000 × g). Subsequently, the extract (20 µL) was taken and introduced to the test-tube and then water (1.58 mL) and Folin – Ciocalteu reagent were added (100 µL). After 5 min the saturated solution of Na_2CO_3 (300 µL) was added. The reaction mixture was incubated under the dim light for 30 min in 40°C. In final step, the absorbance measurements were carried out (3 repetitions) at 760 nm according to the blank probe using the spectrophotometer mentioned above. On the basis of the previously determined calibration curve for ferulic acid, the total content of phenols was estimated in the analyzed plant sample. During the extraction and other stages of the assay, the sample was protected against light and oxygen.

Results and Discussion

The FT-Raman spectroscopy measurements were carried out using the above-mentioned plant species. The variety of forms and species (shrub and herbaceous plants – both of them gathered in their natural habitat, epiphytic tropical fern) were selected so that within each of them it was possible to have plant material of different SLW. The measurements were performed on lyophilized intact leaf blades possessing different SLW values and on tablets obtained from lyophilized and powdered leaves in order to develop a uniform analytical procedure.

Additionally, in order to verify the accuracy of FT-Raman spectroscopic analyses, the biochemical determinations of the total contents of carotenoids and phenols were carried out using the leaves of two selected plant species: *H. bifidum*, representing a plant living in the natural environment, and *P. bifurcatum* grown in greenhouse conditions. For *A. viridis* it was not possible to perform biochemical analyses because of the further unavailability of the plant in its natural conditions, in places where field research was originally carried out.

A. viridis leaves deriving from the locality at an altitude of 568 m a.s.l. (post-agricultural areas, near the village Łobozew – a shadow locality sheltered from the wind) were thin and fragile with SLW = 0.57 g dm^{-2}, Table 1. Oppositely, leaves collected at an altitude of 1215 m a.s.l. (grassland Wetlina, a locality exposed to low temperature, strong wind and intensive sunlight) were smaller and their SLW was significantly higher and amounted to 1.43 g dm^{-2}. In turn, in the case of *H. bifidum* light intensity and spec-

tral composition strongly influenced leaf morphology. The plants growing in high light (not sheltered rock, HL-plants) developed leaves with SLW = 0.88 g dm^{-2}. At the same time, leaves from plants collected from shady localities (localities in the forest, LL-plants) were characterized by significantly smaller SLW equaled 0.40 g dm^{-2}, Table 1.

The nest leaves of *P. bifurcatum* were another tested object. Plants grew under constant light and temperature conditions, therefore the living circumstances were standardized for each specimen. The fact of different SLW values of the base and apex of the green nest leaf is in this case a consequence of the typical differentiation of morphological structure of the leaf blade. SLW for base and apex of the leaf equal 0.59 and 0.20 g dm^{-2}, respectively, Table 1.

Table 1

Specific leaf weight (SLW) parameters (±SD) of *Alnus viridis*, *Hieracium bifidum* and *Platycerium bifurcatum*

Locality/specimen	Specific Leaf Weight (SLW) [g·dm^{-2}]
Alnus viridis	
568 m a.s.l (Łobozew village – LL)	0.57 ±0.11
1215 m a.s.l (grassland Wetlina – HL)	1.43 ±0.13
Hieracium bifidum	
LL-plants	0.29 ±0.04
HL-plants	0.40 ±0.08
Platycerium bifurcatum	
Base of the leaf	0.59 ±0.16
Apex of the leaf	0.20 ±0.04

Explanations: LL-plants – plants with limited access of light; HL-plants – plants that grow in full sunlight

Measurements carried out using FT-Raman spectroscopy on the leaves of three plant species mentioned above allowed the vibration characteristics and distinction of the most important groups of chemical compounds present in all tested leaves, Figure 1 and Table 2. The spectra showed intensive bands typical for carotenoids, observed at frequencies 1005, 1158 and 1525 cm^{-1}, the so-called carotenoid triplet (SCHULZ 2014, VITEK et al. 2017). Each time, the band with a maximum at 1525 cm^{-1} was the most intensive. This band derives from C = C stretching of polyene chain, what also indicates the number of conjugated double bonds in these structures. The obtained values suggest that the main carotenoids present in the tested specimens were lutein, zeaxanthin and β-carotene, all having 9 conjugated double bonds in the molecules (BARANSKI et al. 2005, SCHULZ et al. 2005).

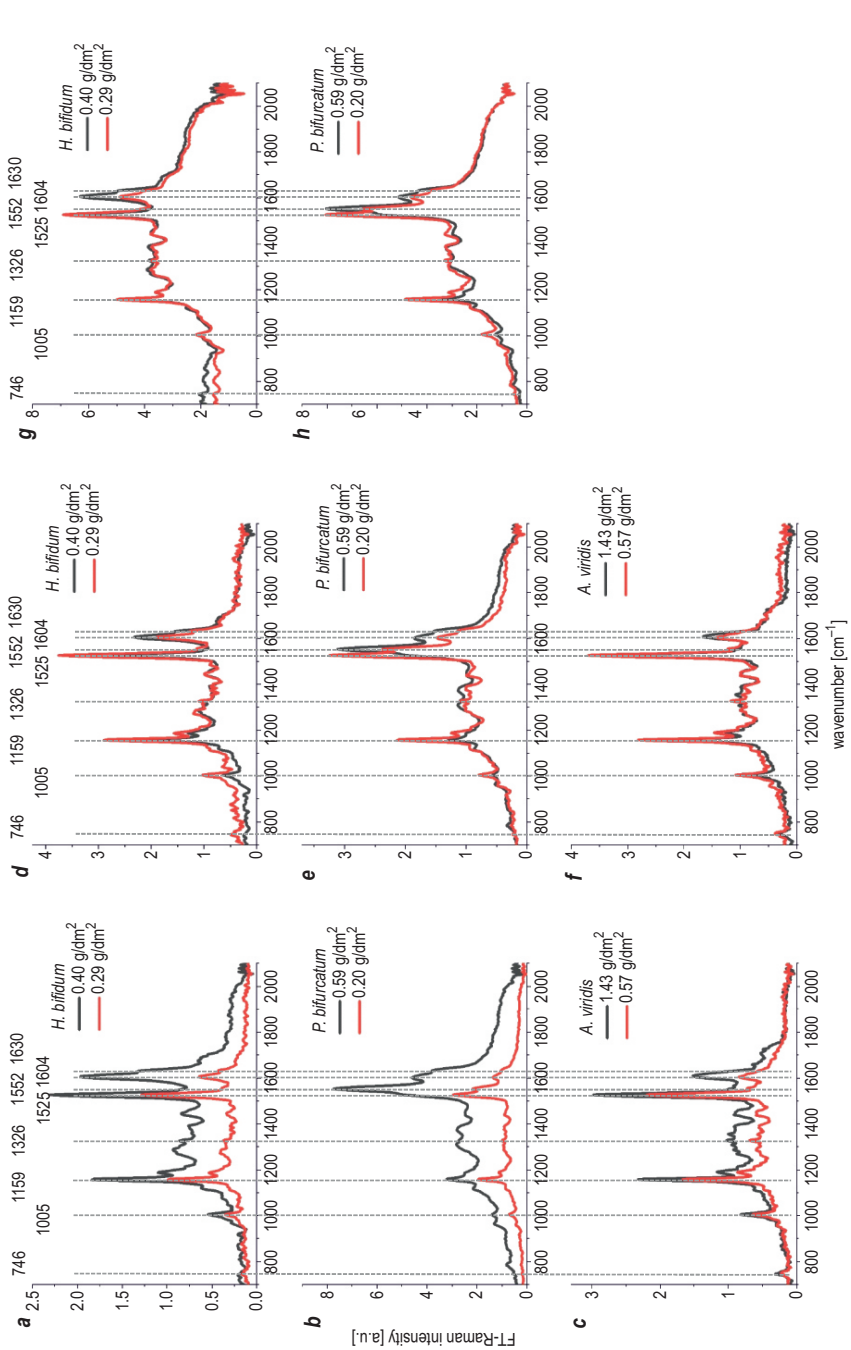

Fig. 1. Spectra of leaves and spectra of tablets: *a*, *b*, *c* – raw FT-Raman spectra obtained for lyophilized leaves of *Hieracium bifidum*, *Platycerium bifurcatum* and *Alnus viridis*, respectively; *d*, *e*, *f* – spectra after normalization according to 1444 cm^{-1}; *g*, *h* – spectra recorded using tablets prepared from lyophilized and powdered leaves of *Hieracium bifidum* and *Platycerium bifurcatum*, respectively. Presented results are the mean values from 4–8 replications

Table 2

Functional groups and vibrational modes obtained for the FT-Raman spectra of tested leaves

Peak number	Wavenumber [cm^{-1}]	Components	References
1	746	chlorophyll	(SCHRADER et al. 1999, VITEK et al., 2017)
2	1005	carotenoid (tetraterpenes), δ(C-CH_3)	(BARANSKA et al. 2005, SCHULZ 2014, VITEK et al. 2017)
3	1159	carotenoids (tetraterpenes) ν(C-C)	(SCHRADER et al. 1999, SCHULZ 2014, VITEK et al. 2017)
4	1189	chlorophyll ν(CC), γ(CH), polyphenols δ(CH)	(ANDREEV et al. 2001, VITEK et al. 2017)
5	1284	poliphenols δ(OH), chlorophyll – δ(CH), ν(CN)	(ERAVUCHIRA et al. 2012, VITEK et al. 2017)
6	1326	chlorophyll – pyrrole ring vibrations, δ(CH), ν(CN)	(BARANSKI et al. 2005, SCHRADER et al. 1998, 1999, VITEK et al. 2017)
7	1525	carotenoids (tetraterpenes) ν(C=C)	(BARANSKI et al. 2005, SCHULZ 2014, SCHULZ and BARANSKA 2007, SCHULZ et al. 2005, VITEK et al. 2017)
8	1552	chlorophyll – central 16-membered ring vibrations, pyrrole ring vibrations, phenolic compounds	(HEREDIA-GUERRERO et al. 2014, PASCAL et al. 2000, SATO et al. 1995, VITEK et al. 2017, ZENG et al., 2021)
9,10	1602, 1630	polyphenols (ν(C-C), ν(C=C) stretching ring)	(HEREDIA-GUERRERO et al. 2014, SCHRADER et al. 1999, SCHULZ and BARANSKA 2007, VITEK et al. 2017)

It should be noted that the band at 1159 cm^{-1}, associated with strong stretching C-C vibrations of the polyene chain arises from the imposition with another, weaker band resulting from deformation vibrations δ (CH) in phenyl rings (from the phenolic compounds) (VITEK et al. 2017). Additionally, at frequencies 746, 1284, 1326 and 1552 cm^{-1} vibrations characteristic of chlorophyll molecules are identified. Furthermore, perhaps the band at 1552 cm^{-1} origins from the superposition of not only chlorophylls but also phenolic compounds (HEREDIA-GUERRERO et al. 2014). In turn, peaks recorded in the range 1250–1400 cm^{-1} indicate the presence of stretching and deformation vibrations specific to the –CH, $-CH_2$ and $-CH_3$

groups building chains of fatty compounds (MUIK et al. 2005, SCHULZ and BARANSKA 2007, THYGESEN 2003). In all the spectra, the bands at 1602 (phenyl-ring stretching mode) and 1630 cm^{-1} (C=C stretching vibration) are also clearly marked, what again confirms the presence of phenolic compounds in the examined plant tissues (VITEK et al. 2017).

Characteristic bands derived from carotenoids, chlorophylls and phenolic compounds (as well as from lipids and fatty acids – not discussed in this work) are visible in Raman spectra recorded directly on the leaves of various plant species, regardless of SLW values. However, SLW values determine Raman signal strength. The greater SLW of the leaf is, the more intensive signal is recorded, Figures 1*a*, *b* and *c*. Therefore, the quantitative comparison of the raw results may not be possible.

Striving to elaborate a reliable procedure for quantitative analysis of the obtained results, the spectra were normalized to the most typical band present in each one at 1444 cm^{-1} (related to C-H vibrations originating most likely from the CH_3, CH_2, and CH functional groups in lipids, amino acid side chains of the proteins, and carbohydrates) (FARBER et al. 2001), Figures 1*d*, *e* and *f*. Following this mathematical operation, spectra of each specimen with different SLW values were found to be largely identical in terms of frequencies and intensities of selected vibrational bands. However, large differences one can find for the bands assigned to carotenoids (1005, 1159, 1525 cm^{-1}) and phenolic compounds (1602, 1630 cm^{-1}). Alternative solution to the problem regarding samples with different SLW was also proposed, namely spectra registered on the previously prepared tablets, Figures 1*g* and *h*. This strategy may also be applied if it is not possible to directly register the spectrum on the plant tissue originating e.g. from shredded herbarium materials. Such an approach can also be an additional method allowing verification of the obtained results. However, one should keep in mind that this requires a time-consuming preparation procedure and leads to destruction of the plant tissue.

Considering the fact that, as it has already been indicated, some bands are the sum of vibrations coming from different components, we decided to decompose the band (registered both for lyophilized leaves – and tablets obtained from lyophilized and powdered leaves of the same plants) in the selected range, precisely 1480–1670 cm^{-1}, Figures 2–4. Peaks coming from C=C (1525 cm^{-1}) vibrations of carotenoid chains and those characteristic of the structure of chlorophylls or phenolic compounds are observed herein. Characteristic bands were selected and the change in peak intensity was analyzed to confirm possible differences in plant tissues (e.g. resulting from different environmental growth conditions). The intensity ratios of selected bands at 1602 to 1525 cm^{-1} (I_{phen}/I_{car}) were compared, Table 3.

Table 3

The Raman intensity ratio (I_{phen}/I_{car}) of peaks belonging to phenols (1602 cm^{-1}) and to carotenoids (1525 cm^{-1}), respectively, obtained after decomposition of FT-Raman bands recorded for *Platycerium bifurcatum*, *Hieracium bifidum* and *Alnus viridis*

	Platycerium bifurcatum			
	SLW = 0.59 [g dm^{-2}]		SLW = 0.20 [g dm^{-2}]	
	leaf	tablet	leaf	tablet
I_{phen}/I_{car}	1.19 ±0.10	0.94 ±0.08	0.30 ±0.03	0.36 ±0.10
	Hieracium bifidum			
	SLW = 0.40 [g dm^{-2}]		SLW = 0.29 [g dm^{-2}]	
	leaf	tablet	leaf	tablet
I_{phen}/I_{car}	0.77 ±0.15	1.01 ±0.03	0.39 ±0.05	0.43 ±0.01
	Alnus viridis			
	SLW = 1.43 [g dm^{-2}]		SLW = 0.57 [g dm^{-2}]	
	leaf		leaf	
I_{phen}/I_{car}	0.36±0.04		0.20±0.04	

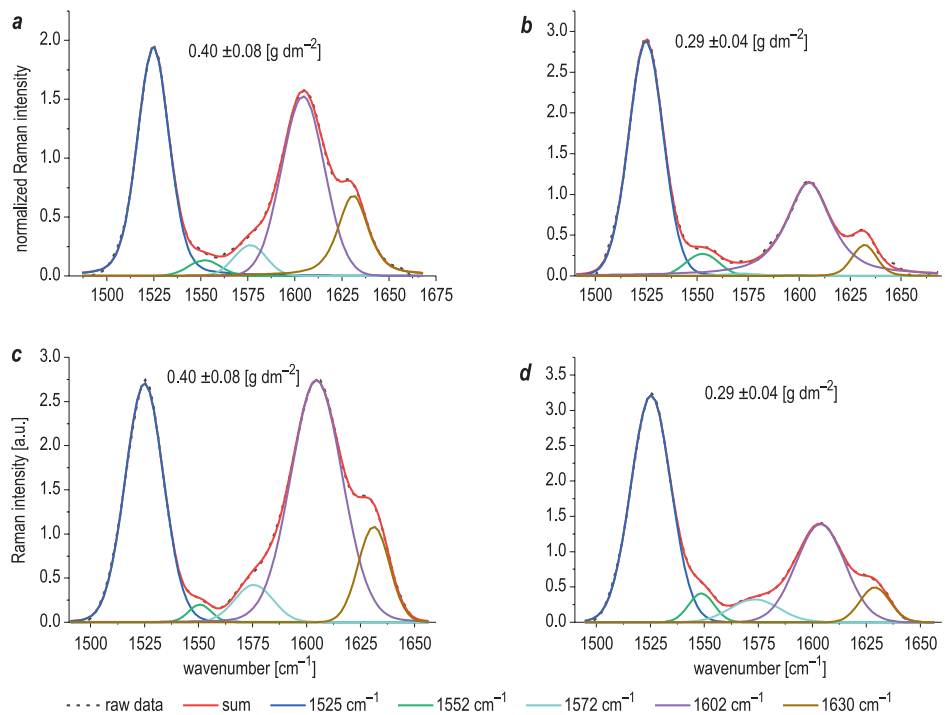

Fig. 2. Decomposition of the recorded FT-Raman spectra of *Hieracium bifidum* performed in the region 1480–1670 cm^{-1}, revealing the quantitative relationships of the phenolic compounds and carotenoids depending on the SLW parameters values: *a*, *b* – decomposition of spectra recorded using leaves with different SLW (values pointed on the pictures); *c*, *d* – decomposition of spectra recorded using tablets prepared from leaves with different SLW (values pointed on the pictures)

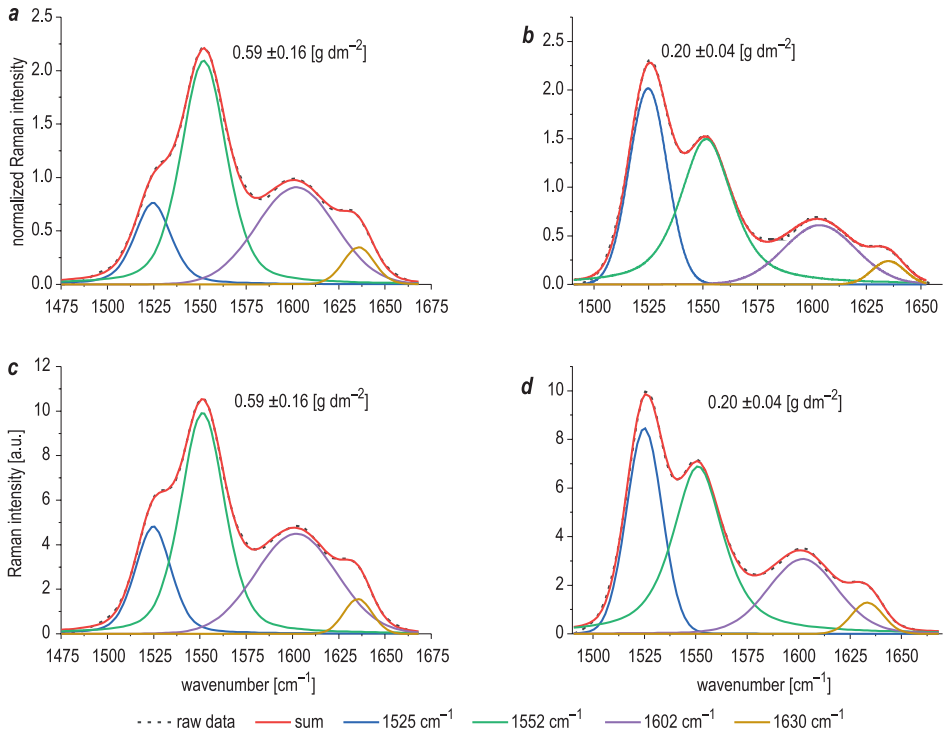

Fig. 3. Decomposition of the recorded FT-Raman spectra of *Platycerium bifurcatum* performed in the region 1480–1670 cm^{-1}, revealing the quantitative relationships of the phenolic compounds and carotenoids depending on the SLW parameters values: *a*, *b* – decomposition of spectra recorded using leaves with different SLW (values pointed on the pictures); *c*, *d* – decomposition of spectra recorded using tablets prepared from leaves with different SLW (values pointed on the pictures)

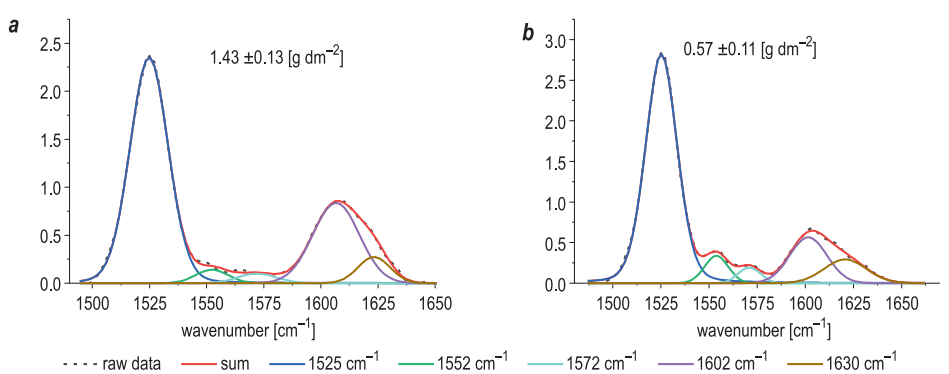

Fig. 4. Decomposition of FT-Raman spectra recorded for leaves of *Alnus viridis* performed in the region 1480–1670 cm^{-1}, revealing the quantitative relationships of the phenolic compounds and carotenoids depending on the SLW parameters values; *a*, *b* – decomposition of spectra recorded using leaves with different SLW (values pointed on the pictures)

This relationship between phenols and carotenoids was analyzed to illustrate quantitative changes that appear under stress factors (light, temperature, wind) affecting the plants. Both carotenoids and phenolic compounds are chemical substances possessing protective activity against stress factors and their contents in plant tissue is strictly connected with external features (QUIDEAU et al. 2011, STRZALKA et al. 2003, TANASE et al. 2019, WEBER et al. 2019).

The obtained results show that the ratios of the intensities of bands at 1602 to 1525 cm^{-1} (I_{phen} /I_{car}) in the spectra of leaves and in the spectra recorded for tablets obtained from powdered leaves of the same investigated plants are consistent what confirms the correctness of the methodology (not only normalization but also the decomposition procedure in order to select the proper bands). Additionally, to verify the results obtained in the spectroscopic investigations, the biochemical analyses of the total phenols and carotenoids were carried out in the case of two tested species: *P. bifurcatum* and *H. bifidum*. The obtained results are set in Table 4. In the case of *P. bifurcatum* the ratios C_{phen}/C_{car} equal 94.85 and 268.18 for leaves characterized with SLW of 0.20 g dm^{-2} and 0.59 g dm^{-2}, respectively. The leaves possessing SLW of 0.20 g dm^{-2} contain more carotenoids and less phenolic compounds. In the case of *H. bifidum* the ratios C_{phen}/C_{car} equal 1184.55 and 3267.49 for the plants growing in low light and high light, respectively (SLW = 0.29 g dm^{-2} and SLW = 0.40 g dm^{-2}).

Table 4

Mean values (±SD) of the contents of phenols and carotenoids assayed for leaves

A	SLW = 0.40 [g dm^{-2}]		SLW = 0.29 [g dm^{-2}]	
	phenols	carotenoids	phenols	carotenoids
Mean values of content [mg/g d.w.]	70.66 ±0.52	0.02 ±0.00	61.95 ±9.01	0.05 ±0.01
C_{phen}/C_{car}	3267.86 ±266.27		1184.55 ±342.27	
B	SLW = 0.59 [g dm^{-2}]		SLW = 0.20 [g dm^{-2}]	
	phenols	carotenoids	phenols	carotenoids
Mean values of content [mg/g d.w.]	134.12 ±38.09	0.52 ±0.20	74.76 ±10.86	0.83 ±0.06
C_{phen}/C_{car}	268.18 ±15.66		94.85 ±15.64	

Explanations: the presented values were obtained for lyophilized leaves of *A* – *Hieracium bifidum* (SLW = 0.40 [g dm^{-2}] and SLW = 0.29 [g dm^{-2}]) and *B* – *Platycerium bifurcatum* (SLW = 0.59 [g dm^{-2}] and SLW = 0.20 [g dm^{-2}]), [mg/g d.w.] – mg of compound on 1 gram of dry weight of leaves; C_{phen}/C_{car} – the ratio of phenols to carotenoids contents. The standard deviations were calculated on the basis of data obtained in 6 series of assays for *B* and 3–4 series of assays for *A*

The leaves of HL-plants possess the lower content of carotenoids and the higher content of phenols in comparison to LL-plants. To conclude, in the case of HL-leaves (HL stress), the total content of phenols is higher whereas the total content of carotenoids is lower in comparison to LL-leaves (shadow conditions). This observation is in line with the results discussed in literature (ALTANGEREL et al. 2017). Shade grown leaves may possess more chlorophylls to increase light capture and less carotenoids and phenolic compounds because of the fact that photoprotection and antioxidant protection are not so demanding (ALTANGEREL et al. 2017, DEMMIGADAMS and ADAMS 1992, TUNGMUNNITHUM et al. 2018). ALTANGEREL et al. (2017) reports a crucial and relevant application of Raman spectroscopy in simultaneous and *in vivo* detection of polyphenols, and carotenoids being reactive oxygen–scavenging pigments. They confirmed the upregulation of phenolic compounds and degradation of carotenoids under abiotic stress (light, drought, salt, cold) occurrence. Additionally, we compared the ratio C_{phen}/C_{car} with the ratio I_{phen}/I_{car} estimated on the basis of the decomposition of FT-Raman spectra recorded directly on leaves and on tablets. Assuming 100% for the leaves with higher SLW parameters, the presented values are a percentage of the same values calculated for leaves of the same species but with lower SLW, Table 5.

We calibrated the FT-Raman method by quantifying carotenoids and phenolic compounds using classical biochemical methods. Therefore, we treat biochemical analyses as an indicator of the actual contents of phenolic and carotenoid compounds and their ratio showing the occurrence in the tissue. For both *H. bifidum* and *P. bifurcatum* the greater similarity of FT-Raman and biochemical results was observed considering the intensity of the peaks obtained by the decomposition of the complex bands registered for tablets (homogenous samples for which we do not observe the effect of SLW on the intensity of the measures Raman signal) instead of leaves, Table 5. This fact confirms the above statement that quantitative interpretation of the results is strongly influenced by tissue morphology. In the case of homogenous material (tablets), the intensities of Raman signals correlate well qualitatively and quantitatively with the ratio of phenols to carotenoids (C_{phen}/C_{car}) for the analyzed leaves, estimated on the basis of the biochemical assays. One should remember that for both tablets (used for FT-Raman measurements) and leaves (used for biochemical analyses) a large amount of plant tissues was employed. If, on the other hand, we make FT-Raman spectra on intact leaf blades, we detect the signal only from its small area. There is one more crucial aspect to be considered regarding the anatomy of the leaf blade. In various leaf blade layers, there is a differing concentration of particular biologically active

compounds (e.i. phenolic compounds are mainly present on the top cell layers of the epidermis whereas chlorophylls and carotenoids are very abundant in chlorenchyma – a deeper layer). As a consequence, we observe a better correlation of the biochemical results with those measured using a homogenous sample (tablet) since powdering the material abolishes these in-depth variations in composition. Therefore, it is the reason for the divergent results obtained between the discussed numerical values for leaves and biochemical determinations, Table 5. This discussed aspect is another limitation that should be considered.

Table 5

Ratios (±SD) of phenols to carotenoids contents estimated on the basis of FT-Raman spectra (leaves and tablets) decomposition (I_{phen}/I_{car}) and biochemical estimations (C_{phen}/C_{car})

Specification	Ratios (±SD) of phenols to carotenoids in [%]	
	Platycerium bifurcatum	*Hieracium bifidum*
I_{phen}/I_{car} (leaves)	25 ±5	51 ±16
I_{phen}/I_{car} (tablets)	38 ±14	42 ±2
C_{phen}/C_{car} (biochem.)	35 ±8	36 ±13

Explanations: the presented values were calculated for leaves with a lower SLW and are a percentage of the same values obtained for leaves with a higher SLW (100%)

In order to discuss the Raman spectra, many researchers use chemometric methods (e.g. cluster analysis), the purpose of which is to find significant and systematic differences in the recorded spectra (RYS et al., 2020). However, taking into account the fact of different SLW of leaves of the same species, cluster analysis should not be performed directly on the raw data obtained from FT-Raman measurements. First, the spectra should be averaged, normalized, and finally they might be further transformed (cluster analysis) (PAYNE and KUROUSKI 2021). Otherwise, we may run the risk of receiving erroneous results of the analyses performed.

In conclusion, it should be emphasized that the quantitative comparison of the results obtained for morphologically different leaves, with significantly different SLW values, is not possible.

Conclusions

The goal of our research was to investigate to what extent the differences in SLW of the leaves of the same species allow us to reliably determine qualitative and quantitative changes in their chemical composition, using FT-Raman spectroscopy. For the experiments, we used lyophilized leaves of three plant species (*A. viridis*, *H. bifidum*, *P. bifurcatum*).

Based on the obtained results, we proved that if we employed the FT-Raman spectroscopy method to study changes in the chemical composition of plant tissues subjected to stress factors, influencing the morphology of leaves, namely their SLW, direct comparison of the obtained results might be difficult or even impossible.

In such cases, it is suggested to normalize the spectra (which have already been presented and reported many times). It is necessary to remember that this mathematical calculation should be performed considering the one particular band that originates from vibrations, typical for all tested samples (e.g. aliphatic). The normalization procedure is necessary for chemometric analysis (cluster analysis), otherwise, the obtained results will not be reliable. Raman spectra performed on the leaf blade allow for the detection of the signal only from the small, specified area. Thus, if we want to study the global impact of stress on the tissue, the normalization procedure is not enough. The solution to this problem is preparing a homogeneous sample, e.g. in the form of a tablet obtained from lyophilized and powdered leaves. This procedure allows for the unification and standardization of samples and avoids the influence of SLW (in practice the thickness of the leaves) on the results of quantitative analysis. One should also take into account the overlap of peaks coming from vibrations characteristic for the analyzed chemical groups. In such a situation, normalization is not enough to obtain quantitative results. It is necessary to apply additional mathematical operation – decomposition, which allows to isolate the components of a given band responsible for the analyzed vibrations.

Summarizing, the application of FT-Raman spectroscopy in research into plants is indisputably essential and beneficial, however in case of inhomogeneous tissues particular limitations of this method should be considered and a special procedure of analyzing the spectra should be taken.

Conflict of interests. The authors declare no competing interests.

Accepted for print 26.03.2024

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