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Changes in physiological features of undergrowth indicator species of old forest

Introduction

The small remnants of old forests are currently the only local refuges for many species of plants and animals. Observations in various parts of Europe, as well as in Poland, have shown that in these remnants of old forests there are usually many more typical forest species than in young forests and tree plantations, overgrowing or planted on unused fields, meadows and grasslands (Peterken, Game, 1984; Dzwonko, Loster, 1989; Wulf, 1997; Hermy et al., 1999; Orczewska, 2010; Matuszkiewicz et al., 2013). Forest species colonising isolated new forests slowly or unable to do so can be considered as indicators for old forests. Their more numerous presence indicates a long and uninterrupted existence of a forest habitat in a given place. Over 150 species of vascular plants are at least regional indicators of old forests in Poland (Dzwonko, Loster, 1988; 2001). Among them are such forest plants as: *Anemone nemorosa* L., *Asarum europaeum* L., *Convallaria majalis* L., *Galeobdolon luteum* Huds. emend. Holub, *Galium odoratum* (L.) Scop., *Majanthemum bifolium* (L.) F.W. Schmidt, *Oxalis acetosella* L., *Stellaria holostea* L. They were so common until recently and are still common in some areas. According to Kimberley et al. (2013) the following features distinguish old forest indicator species from other forest species: lifespan (perennial species), short stature, fast-dropping, heavy seeds, poor dispersal, low tolerance for severe disturbance and high habitat productivity.

In the modern lowland and foothill landscape of Poland, the remnants of deciduous and mixed forests of natural origin usually occupy isolated areas. The "Las Wolski" For-

est, located within the administrative borders of Cracow (Southern Poland) is a good example. This has an area of 419 hectares and plays important recreational functions for the city of Cracow (Dubiel, 1971; Rutkowski, 1984). The landscape is diversified, with gorges and limestone steep rock walls. The soil substrate is loess forming a thick layer on the tops, and in the ravines there are also rendzinas with a poorly developed profile (Zygmunt et al., 2014). Forest communities building Las Wolski include oak-pine forest *Querco roboris-Pinetum* (W. Mat. 1981) J. Mat. 1988 (with two variants: with *Fagus silvatica* L. and typical), hornbeam forest *Tilio-Carpinetum* Tracz. 1962 and coniferous trees plantation on former hornbeam sites. The occurrence of hornbeam forest in Las Wolski is limited to the edges of the plateau and shady ravines. The flora of the forest has been thoroughly studied (e.g. Berdau, 1859; Krupa, 1877; Raciborski, 1884; Kornaś, 1948; Kornaś, Medwecka-Kornaś, 1974, 2011; Zygmunt et al., 2014). In some studies, attention has long been paid to changes in the flora related to human activity. Many plant species that formerly existed in the Wolski Forest no longer grow there (e.g. *Anemone silvestris* L., *Blechnum spicant* (L.) Roth., *Huperzia selago* (L.) Bernh. ex Schrank et Mart.) and many others occurring in great numbers have become a great rarity (e.g. *Corydalis cava* Schweigg. et Koerte, *Galanthus nivalis* L. and others). The growing human pressure is the main driving factor of habitat changes in Las Wolski (Poznański, 2014; Banach, Skrzypek, 2018).

The height of a plant in relation to its neighbour, especially in forest, determines the quantity and quality (spectral composition) of light, which is shaped by a whole set of environmental factors, such as changes in temperature, wind intensity, relative air and soil humidity (Franklin, Whitelam, 2005; Hetmann, Kowalczyk, 2011; Keller et al., 2011; Kusior et al., 2012; Kirchhoff, 2014). The crowns of the tallest forest trees always receive light with a full spectral composition, starting from violet light, through blue, red and far red light, while a small amount of blue and red light and a large amount of far red light reach the forest floor (Kwak et al., 2011; Casal, 2012; Pilarski et al., 2012). The structure of the forest, a path of light penetration, an angle of incidence of sunlight, weather conditions, a season of the year, a month or even a day all influence the lighting conditions, which are variable in time and space (Endler, 1993; Théry, 2001; Pilarski et al., 2012). Changes in lighting conditions can also be initiated by habitat changes resulting from the human influence. Gaps in the tree stand, intensive trampling, especially in recreational forests, can be a direct cause of changes in lighting, which adversely affect undergrowth species, including those indicative for old forests.

The physiology of indicator species of old forests is still poorly understood. Therefore, the aim of this study was to investigate selected physiological features of three indicator species of the undergrowth of the hornbeam forest, after the foliage was closed in the tree layer.

Material and methods

Plant material and habitat

Our study concerns: wood anemone *Anemone nemorosa* L., yellow archangel *Galeobdolon luteum* Huds. emend Holub (syn. *Lamium galeobdolon* (L.) Crantz) and greater stitchwort *Stellaria holostea* L. (syn. *Rabelera holostea* (L.) M.T. Sharples & E.A. Tripp).

Fig. 1. Sites of harvesting plant material

Two sites for plant material collection were located in the hornbeam stands within Las Wolski forest complex (50°03′21.7 3′N 19°50′51.27′E), located in the western part of Cracow (Fig. 1). The collection sites were characterised using the phytoindication method, according to Ellenberg values (Ellenberg et al., 1992). Based on the floristic lists of undergrowth species with the determination of their quantitative share on the areas (100 m^2 each), weighted averages (in terms of quantity) of the following indicates were calculated: light – L, soil moisture – F, soil pH – R and soil nitrogen – N (Tab. 1).

Tab. 1. Habitat characteristics of the study sites in Las Wolski based on weighted average values of Ellenberg indicators (light, soil moisture, soil pH, soil nitrogen)

Site No.	Average values of indicators according to Ellenberg ±SD			
	L (light)	F (soil moisture)	R (soil pH)	N (soil nitrogen)
1	4.06 ±1.03	5.30 ±0.51	7.26 ±0.48	6.50 ±1.36
2	4.69 ±1.19	5.30 ±0.47	6.46 ±1.28	5.76 ±1.29

The analysis carried out here shows that these areas are dominated by shade-loving species, preferring fresh, slightly acidic and medium fertile soils.

Wood anemone and yellow archangel were collected at site number 1, greater stitchwort was sampled at site 2. Five individuals per species, along with associated soil (without damaging the entire root ball), were collected on June 22, 2020, immediately after the full development of the forest canopy and again on July 22, 2020, one month after the closure of the forest canopy, at the stage of maximum tree leaf development. The harvest was done in the early afternoon at 20°C (22/06) and 23°C (22/07) and moderate sunshine. After collection, the material was transported to the laboratory and immediately subjected to all analyses in laboratory conditions.

Biomass analysis

The fresh mass of individual fresh cut leaves of the tested species was determined on a laboratory scale with an accuracy of 0.0001 g (Ohaus Adventurer Pro, Parsippany, New Jersey, USA). The weighed fresh leaves were placed in Petri dishes (ø 3 cm) with 5 ml of distilled water so that the petioles were immersed in the water, and left for 24 h. After this time, the leaves of the studied species were weighed again. The dry mass was obtained after drying the plant material in a dryer (WAMED SUP 100, Poland) at 105°C for 48 hours. Based on the obtained results, the relative water content (RWC) and the total tissue water content (TWC) of individual leaves were determined according to the following formulas (Barrs, 1968):

$$RWC\ (\%) = [(FM - DM)/(TM - FM)] \times 100,$$

where: RWC – relative water content, FM – fresh mass, DM – dry mass, TM – fresh mass of the leaf after 24 hours of leaving it in 5 ml of distilled water (turgor) (Hura et al., 2014; Kwaśniewski et al., 2016),

$$TWC\ (\%) = [1 - (DM/FM)] \times 100,$$

where: DM – dry mass, FM – fresh mass (Hellal et al., 2018).

Electrolyte leakage

The percentage of electrolyte leakage was tested according to the method used in the work of Możdżeń et al. (2020). Single leaves of the analysed plant species were placed in polypropylene falcons with 30 ml of distilled water, with conductivity of 0.05 μS. To measure electrolyte leakage from living leaves (EC_1), each vial was shaken for 3 hours on a shaker (Labnet, Rocker, New Jersey, USA). The degree of destabilisation of cell membranes in living cells was measured using a conductivity meter (CX-701, Elmetron, Zabrze, Poland), with an electrode with constant K = 1.02 (Elmetron, Zabrze, Poland). After the first measurement, leaves in distilled water were frozen at –75°C for 24 hours to macerate the material. Subsequently, the samples were thawed and subjected to the same procedures as described above, and the amount of dead leaf electrolyte leakage (EC_2) was determined. Based on the obtained results, the percentage leakage of electrolyte was determined according to the following formula:

$$EL = (EC_1 / EC_2) \times 100,$$

where: EL – percentage of electrolyte leakage, EC_1 – electrolyte leakage in living cells, EC_2 – electrolyte leakage from dead cells.

Chlorophyll content

Total chlorophyll content was measured using a SPAD chlorophyll meter. The content of chlorophyll *a* and *b* was determined using the spectrophotometric method (Barnes et al., 1992). Discs with a diameter of 1 cm were cut from the leaves of the studied species, weighed (scales – Ohaus Adventurer Pro, Parsippany, New Jersey, USA) and extracted in 3 ml of dimethyl sulfoxide (SIGMA-Aldrich, St. Louis, MO, USA) for 48 h at 65°C. The chlorophyll extract was poured into 1 ml polypropylene cuvettes and measured at two wavelengths λ = 648 and 665 nm on a spectrophotometer (CECIL, United Kingdom).

Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence was measured using a fluorimeter (Hansatech, King's Lynn, United Kingdom). In order to extinguish the light phase of photosynthesis, clips blocking light were placed on the tested leaves for dark adaptation 30 minutes, prior to conducting the test. After this time, the leaves were exposed to the excitation light of 1000 μmol × $m^{-2} \times s^{-1}$ for 1 s and the following fluorescence parameters were determined: F_0 – zero fluorescence, F_m – maximum fluorescence, F_v – variable fluorescence, F_v/F_m – maximum photochemical efficiency PSII (Lichtenthaler et al., 2004a).

In order to visualise the fluorescence of chlorophyll *a*, the leaves of the tested species were spread on filter paper soaked in distilled water and adapted to the dark for 30 minutes in a closed measurement chamber FluorCam FC 800C (Photon Systems

Instruments, Czech Republic) (Lichtenthaler et al., 2004a). From the obtained results, F_t – stationary fluorescence, NPQ – non-photochemical quenching and Rfd – PSII vitality index were analysed.

Chlorophyll fluorescence emission

The blue-green and red fluorescence emission spectra were measured on a spectrofluorometer (Perkin-Elmer LS55B, UK), according to the method of Lichtenthaler et al. (2004b). Fluorescence intensity in the blue-green (430–650 nm) range was measured at 390 nm excitation as well as near and far red (650–800 nm), with 430 nm blue excitation. The gap for the excitation ray was 15 nm, and for the emitted ray 20 nm. Fluorescence intensity indices were determined on the basis of the spectra F440/F535, F440/F595, F440/F695, F440/F735, F695/F735. The results were analysed using FL WinLab version 3.00. On the basis of the methodology used by Jena et al. (2012), the activity of the cortical (C) and antenna (A) parts of the PSI and PSII systems were determined.

Statistical analysis

The results were obtained from 5 repetitions for each of the tested objects in two dates. Statistica 13.0 software was used in statistical calculations. To determine the differences between the obtained experimental values, the standard deviation (±SD) was calculated for each parameter and the Duncan test for independent samples was used ($p < 0.05$).

Results

The fresh mass of individual leaves was significantly higher for plants collected in June than in July for both *G. luteum* and *A. nemorosa* (Tab. 2).

Tab. 2. Mass and water content of leaves of indicator plants collected on two dates (A) – June 22, 2020 and (B) – July 22, 2020, in Las Wolski; numbers represent mean values ±SD (n = 5); different lower case letters represent significant differences at $p \leq 0.05$ according to Duncan's test

Parameter	Galeobdolon luteum		Stellaria holostea		Anemone nemorosa	
	A	B	A	B	A	B
Fresh mass (mg)	0.1218 a ±0.04	0.0768 b ±0.01	0.0378 a ±0.01	0.0331 a ±0.01	0.2513 a ±0.08	0.1043 b ±0.02
Dry mass (mg)	0.0192 a ±0.01	0.0135 a ±0.002	0.0059 a ±0.01	0.0069 a ±0.002	0.0407 a ±0.01	0.0187 b ±0.01
RWC (%)	95.56 a ±2.79	92.61 b ±1.67	84.71 a ±4.25	79.08 a ±13.27	88.49 a ±2.42	76.65 b ±8.82
TWC (%)	84.12 a ±1.34	82.39 a ±2.50	84.32 a ±0.84	79.04 b ±1.44	83.94 a ±0.09	82.18 a ±1.61

RWC – Relative Water Content (turgor), TWC – Total tissue Water Content

No significant difference in the fresh mass between the collection dates was found in the case of *S. holostea*. The dry mass of *G. luteum* and *S. holostea* leaves did not differ statistically between the analysed harvest dates. For *A. nemorosa*, a decrease in the dry mass value for leaves from the second measurement period was observed. The relative water content (RWC) in the leaves of *G. luteum* and *A. nemorosa* was higher in the first period of measurements compared to the second period. *S. holostea* showed no significant changes in RWC values. The total water content of leaves of *G. luteum* and *A. nemorosa* did not differ significantly between harvest dates. In the case of *S. holostea*, a significant decrease in the water content (TWC) in the leaves from the second measurement period was found in relation to the value of this parameter in the first period of the study.

Plasma membranes integrity was determined by measuring electrolyte leakage (EL). The degree of destabilisation of *G. luteum* leaf cell membranes did not differ significantly between the first and second term (Fig. 2). In the case of *S. holostea*, an increase in electrolyte leakage was observed in the second period of harvest, compared to the first period of analysis. For *A. nemorosa*, the percentage of electrolyte leakage was higher in the first harvest period than in the second measurement period.

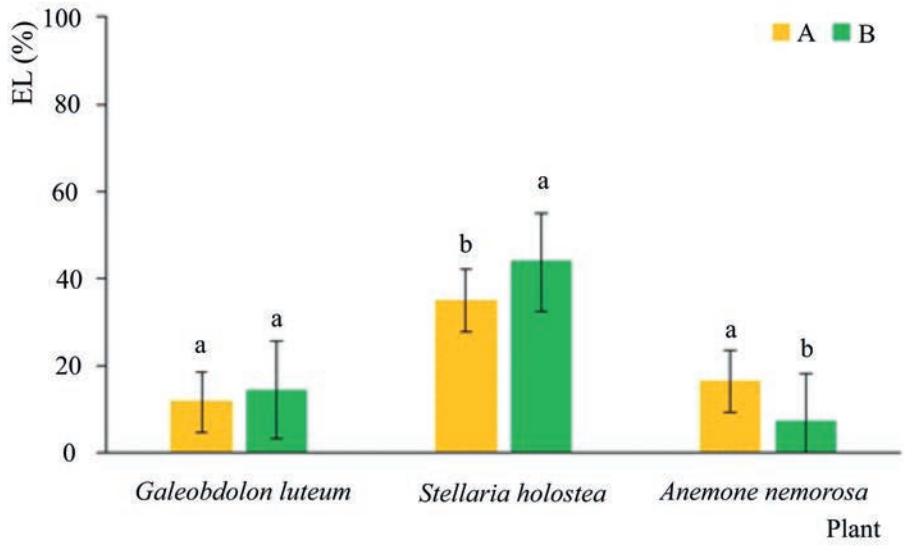


Fig. 2. Electrolyte leakage (EL) from leaves of indicator plants collected on two dates (A) – June 22, 2020 and (B) – July 22, 2020, in Las Wolski; mean values ±SD (n = 3); different letters represent significant differences ($p \leq 0.05$) within each species according to Duncan's test

Statistical analysis of the total chlorophyll content showed significant differences in the concentration of this group of dyes for each of the studied species (Fig. 3). In the case of *G. luteum* and *A. nemorosa*, a higher concentration of chlorophyll was found

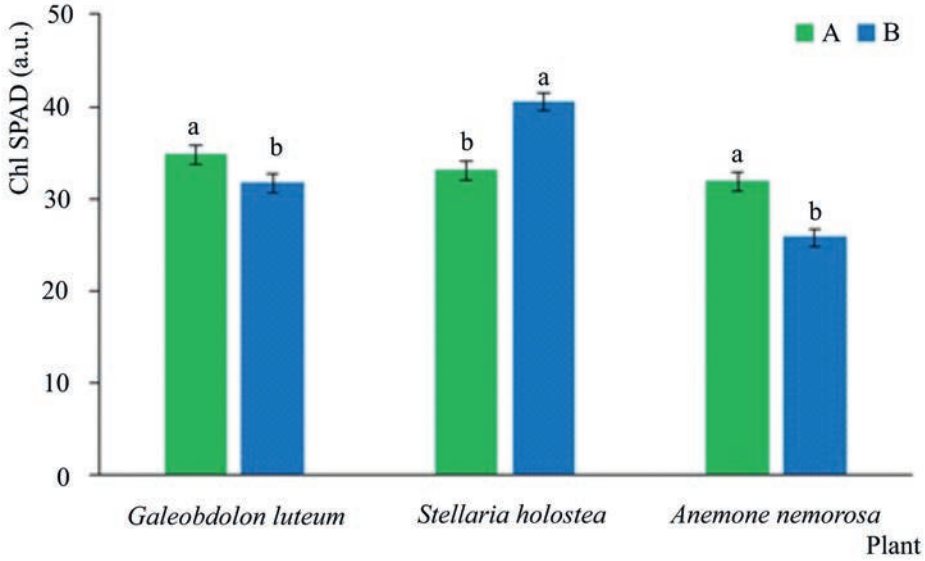


Fig. 3. Total chlorophyll content measured with the SPAD chlorophyll meter of indicator plants collected on two dates (A) – June 22, 2020 and (B) – July 22, 2020, in Las Wolski; mean values ±SD (n = 3); different letters represent significant differences ($p \leq 0.05$) within each species according to Duncan's test

in the leaves of plants harvested in the first period, than in the second period of measurements. For *S. holostea*, a lower chlorophyll content was observed in the first harvest period compared to the second harvest period.

The studies of the content of chlorophyll *a*, *b* and total content (*a* + *b*) carried out between the first and second dates of plant harvesting differed statistically (Tab. 3).

Tab. 3. Chlorophyll content (Barnes method) in the leaves of indicator plants collected on two dates (A) – June 22, 2020 and (B) – July 22, 2020, in Las Wolski; mean values ±SD (n = 3); different letters represent significant differences ($p \leq 0.05$) within each species according to Duncan's test

Chlorophyll	*Galeobdolon luteum*		*Stellaria holostea*		*Anemone nemorosa*	
	A	B	A	B	A	B
a	2.54 a ±0.29	1.03 b ±0.20	1.80 b ±0.15	2.98 a ±0.12	1.48 a ±0.06	0.88 b ±0.04
b	0.87 a ±0.09	0.57 b ±0.06	0.57 b ±0.03	0.97 a ±0.04	0.62 a ±0.04	0.30 b ±0.03
a + *b*	3.41 a ±0.37	1.60 b ±0.24	2.37 b ±0.17	3.95 a ±0.19	2.10 a ±0.09	1.18 b ±0.06

A higher concentration of chlorophylls in the first harvest period was found in the leaves of *G. luteum* and *A. nemorosa*. Opposite data were obtained for *S. holostea* plants, where the concentration of chlorophylls was higher in the second period of measurements, in relation to the first harvest date.

Measurements of chlorophyll *a* fluorescence showed no differences in the values of initial fluorescence (F0) in the tested species. In the case of maximum fluorescence (Fm), a significantly higher value of this parameter was noted for *S. holostea* in the second measurement date (Tab. 4).

Tab. 4. Chlorophyll *a* fluorescence parameters of indicator plants collected on two dates (A) – June 22, 2020 and (B) – July 22, 2020 in Las Wolski; mean values ±SD (n = 3); different letters represent significant differences ($p \leq 0.05$) within each species according to Duncan's test

Species	F_0		F_m		F_v		F_v/F_m	
	A	B	A	B	A	B	A	B
Galeobdolon luteum	293 a ±29.74	306 a ±41.35	1772 a ±155.47	1832 a ±276.68	1479 a ±125.90	1526 a ±235.66	0.834 a ±0.003	0.832a ±0.004
Stellaria holostea	238 a ±25.00	264 a ±27.75	1467 b ±168.11	1810 a ±203.94	1228 b ±144.46	1543 a ±178.15	0.837 a ±0.010	0.854 a ±0.003
Anemone nemorosa	273 a ±56.62	267 a ±51.46	1477 b ±273.11	1362 a ±300.11	1204 a ±217.79	1094 a ±251.00	0.816 a ±0.010	0.802 a ±0.010

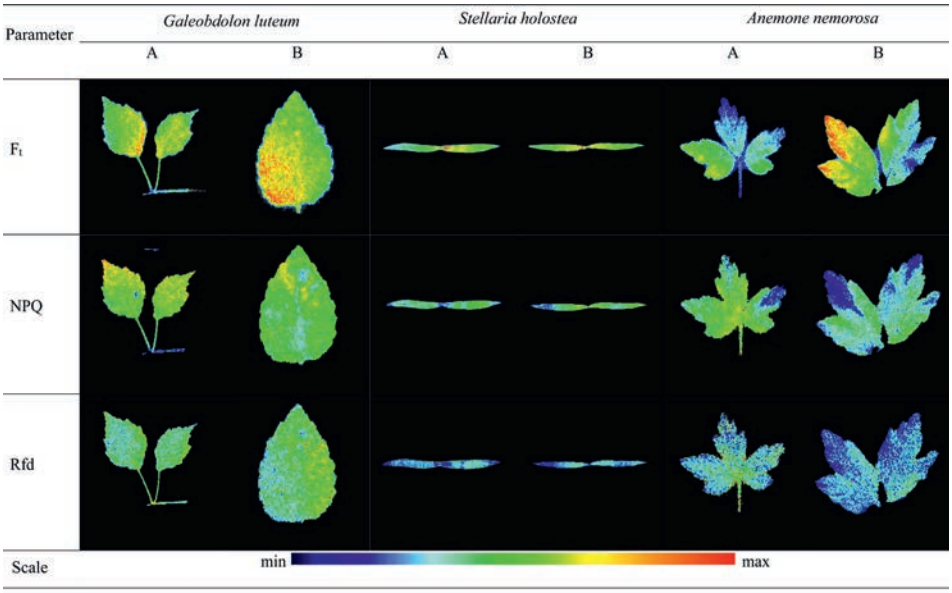
F_0 – initial fluorescence (zero), F_m – maximum fluorescence, F_v – variable fluorescence, F_v/F_m – photochemical photon yield in PSII

For the remaining two species, there were no statistically significant differences in F_m values. The variable fluorescence (F_v) reached significantly the highest values in *S. holostea* on the second measurement date, o significant differences in F_v. The fluorescence parameter of photochemical photon yield in PSII (F_v/F_m) was statistically insignificant for *G. luteum* and *A. nemorosa*. Differences in F_v/F_m values were found for *S. holostea* between the first and second date. Higher PSII activity of *S. holostea* leaves was observed in the second period of measurements.

Imaging of chlorophyll *a* fluorescence parameters of the leaves of the studied species allowed for the presentation of the photosynthetic activity of the entire leaf blade in a given vegetation period (Tab. 5). For *G. luteum*, in the first and second time of harvest, the stationary fluorescence – in the stationary phase of photosynthesis (F_t) was similar over the entire surface of the leaves (green, alternating with yellow and red). Only on the edges of the leaf blade, for both measurement dates, lower F_t values (bright blue) were observed. Non-photochemical quenching (NPQ) in *G. luteum* leaves was higher in the first harvest date in the upper part of the leaf blade (yellow and red). In the second time of measurements, the activity of this parameter was lower (blue and green). The PSII vitality index (Rfd) in the first measurement period was slightly lower (more blue) than in the second analysis period, where yellow spots appeared. For *S. holostea*, stationary fluorescence Ft was lower in the first measurement period than in the second one. In the first period, blue and green colours dominated, and in the second, yellow and red colours occurred on a larger surface of the leaf blade. The NPQ and Rfd values in this species were similar between the measurement dates. In the case of *A. nemorosa*,

the F_t parameter reached higher values in the second measurement period (red and yellow) than in the first one. The NPQ and Rfd parameters for wood anemone were lower in the second term (more blue).

Tab. 5. Imaging of chlorophyll *a* fluorescence of leaves of indicator plants collected on two dates (A) – June 22, 2020 and (B) – July 22, 2020, in Las Wolski



F_t – stationary fluorescence, NPQ – Non-photochemical quenching, Rfd – PSII vitality index

The shape of the fluorescence emission spectra in the blue range, both in the first and second time of measurements of the leaves of the studied species, was similar (Fig. 4). The first peak was found at around 530 nm and the second one at 590 nm. Higher values were recorded for all analysed leaves in the first period of measurements, in relation to the second date of harvest. In the red range, the fluorescence emission of the leaves of the studied species had a similar shape of the spectra. Two clear peaks were observed at around 690 nm and 735 nm in the spectra of all the tested species. For *G. luteum*, higher fluorescence emission values were found in the first measurement period. Similar emission values between the first and second measurement dates were found for *S. holostea*, and slightly higher in the second date for *A. nemorosa*.

The F440/F535 coefficient did not differ significantly in any of the studied species between harvest dates (Tab. 6). In the case of F440/F695 and F440/F735 coefficients, a significantly lower values were found in the second measurement date only in the leaves of *G. luteum*. In the leaves of *G. luteum* and *A. nemorosa*, the value of the F440/F595 coefficient was statistically lower in the first period of measurements in relation to the second period.

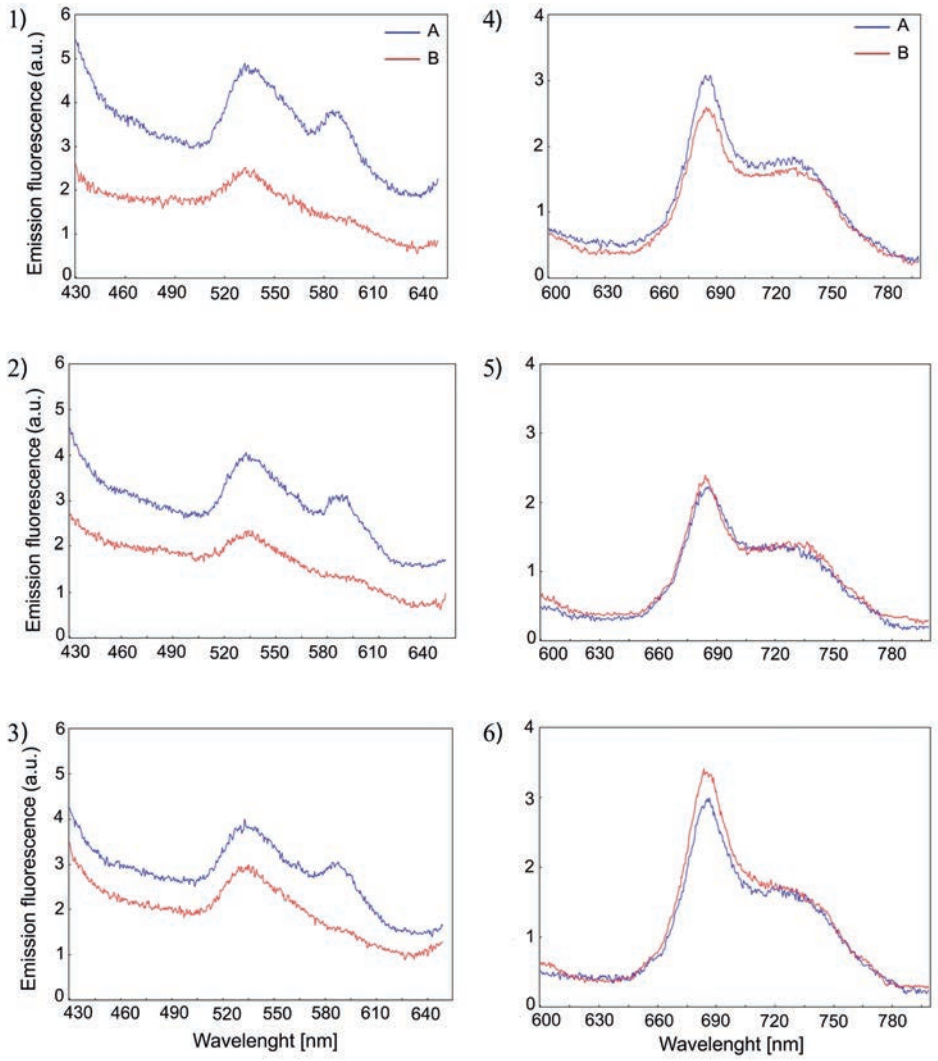


Fig. 4. Fluorescence emission spectra of leaves of indicator plants collected on two dates (A) – June 22, 2020 and (B) – July 22, 2020, in Las Wolski; blue range of spectra: 1 – *Galeobdolon luteum*, 2 – *Stellaria holostea*, 3 – *Anemone nemorosa*, red range of spectra: 4 – *G. luteum*, 5 – *S. holostea*, 6 – *A. nemorosa*

The values of F696/F735, PSIA/PSIC, PSIIA/PSIIC in the three species studied were similar and did not differ between harvest periods. In the case of the PSI/PSII coefficient, its lower values were recorded in the first harvest period only in the leaves of *S. holostea*. In the remaining two species, no differences in the values of this coefficient were found.

Tab. 6. Fluorescence emission coefficients of leaves of indicator plants collected on two dates (A) – June 22, 2020 and (B) – July 22, 2020, in Las Wolski; mean value ±SD (n = 3); different letters represent significant differences ($p \leq 0.05$) within each species according to Duncan's test

Fluorescence emission factor	*Galeobdolon luteum*		*Stellaria holostea*		*Anemone nemorosa*	
	A	B	A	B	A	B
F440/F535	0.97 a ±0.05	0.94 a ±0.04	0.94 a ±0.10	1.03 a ±0.04	0.90 a ±0.05	0.90 a ±0.02
F440/F695	2.02 a ±0.28	1.16 b ±0.14	2.16 a ±0.56	1.46 a ±0.20	1.50 a ±0.15	1.08 a ±0.39
F440/F735	2.54 a ±0.26	1.37 b ±0.11	3.07 a ±0.89	1.76 a ±0.12	2.17 a ±0.36	1.67 a ±0.59
F440/F595	1.33 b ±0.05	1.58 a ±0.06	1.35 a ±0.11	1.70 a ±0.13	1.24 b ±0.04	1.76 a ±0.30
F695/F735	1.26 a ±0.04	1.18 a ±0.08	1.41 a ±0.11	1.21 a ±0.10	1.44 a ±0.10	1.56 a ±0.12
PSIA/PSIC	1.35 a ±0.03	1.35 a ±0.06	1.27 a ±0.04	1.44 a ±0.11	1.29 a ±0.06	1.33 a ±0.03
PSIIA/PSIIC	1.00 a ±0.06	1.02 a ±0.05	1.10 a ±0.07	1.02 a ±0.04	1.06 a ±0.11	1.05 a ±0.03
PSI/PSII	1.35 a ±0.08	1.33 a ±0.08	1.16 b ±0.07	1.42 a ±0.12	1.23 a ±0.12	1.27 a ±0.04

Discussion

The basic requirement for plant tolerance to changes in habitat parameters is the quick reception of signals from the external environment and the so-called adaptation decisions, involving the launching or altering the developmental programs influencing the course of life processes. This includes the coordination between the production of nutrients and their distribution throughout the plant body. Stress imposes the need to run energy requiring processes related to the acclimatisation and adaptation and limits photosynthetic production (e.g. Starck et al., 1993; Starck, 2010).

During the conducted research, a decrease in the value of the leaf fresh mass of the analysed indicator species was noted in the second study period, when maximum tree foliage development should be expected (Tab. 2). The obtained result may be the result of natural phenological transformations, typical for the undergrowth species of the deciduous forests. *Anemone nemorosa* is an early spring plant, used also in the determination of the phenological stage of the forest (Molga, Sokołowska, 1963). It is a rhizomatous geophyte that blooms in March and April and bears fruit in May. At the beginning of summer, its above-ground parts die, and the plant enters a state of rest (*Flora Polski*, www.atlas-roslin.pl). *Galeobdolon luteum* and *Stellaria holostea* are chamaephytes, flowering and fruiting from May to June. *G. luteum* may have partially evergreen leaves. Thus, the lower mass values recorded here may be the effect of phenological changes, adapted to the rhythmic changes of light conditions, caused by the growth of leaves of the canopy trees (Théry, 2001; Pilarski et al., 2012).

Shading caused by foliar development of the overlying forest layers can be an environmental stress for the undergrowth plants (Endler, 1993; Théry, 2001). For example, when comparing the leakage of electrolyte from the leaf cells of the analysed species as a measure of environmental stress (Dexter et al., 1932; Bajji et al., 2002), higher values of this parameter were obtained for *A. nemorosa* in the first study period (just after reaching tree foliage density), and the results for *S. holostea* were opposite (Fig. 2). *Anemone nemorosa* is a shade tolerant species (*Flora Polski*, www.atlas-roslin.pl). Although it blooms and bears fruit before the tree canopy closes, the increased amount of light reaching its leaves can act as a stress factor. In contrast, in case of *S. holostea* may act the other way around. Forest canopy foliage closes restricting the access of light, may be a stress factor for this plant. In the case of *A. nemorosa*, it seems interesting because in conditions of increased environmental stress, this species blooms and bears fruit, also achieving higher parameters of fresh and dry mass (Tab. 2).

Changes in the content of assimilation pigments noted during the studies carried out here may also reflect changes in phenology (Pilarski et al., 2012). Both the content of chlorophyll measured with the SPAD chlorophyll meter (Fig. 3) and the content of chlorophyll *a* and *b* determined by the Barnes method (Tab. 3) showed that in the case of *A. nemorosa* and *G. luteum*, at the maximum stage of tree foliage closure, there was a significant reduction in the content of assimilation dyes. The opposite situation was noted for *S. holostea*. The reduction of these parameters in *A. nemorosa* and *G. luteum* can be treated as the preparation of these plants for dormancy after flowering and fruiting, additionally enhanced by the effect of shadow. Explaining the increase in the content of pigments found in *S. holostea* is much more difficult. Probably some other environmental or species related factors that are not yet sufficiently understood influence this process. Perhaps this is related to the statistically significant increase in the maximum fluorescence (Fm) and the variable fluorescence (Fv) noted here (Tab. 4). In the case of maximal fluorescence, induced by a saturating light pulse after dark adaptation, all PSII reaction centres are extinguished, as this is a state of saturation with light (Baker, 2008). Lower values of this parameter may indicate some kind of stress (Sulkiewicz, Ciereszko, 2016), which may result in the fact that not all electron acceptors in PSII are completely reduced (Kalaji, Łoboda, 2010).

The efficiency of photosynthesis can be an excellent indicator of the condition of plants in specific habitat conditions (Lin, Jolliffe, 2000; Oxborough, 2004). The most commonly used parameter to assess the maximum efficiency of PSII is the ratio of variable fluorescence to maximum Fv/Fm. The value of this parameter for vascular plants should not be lower than 0.80; optimally around 0.83 (Björkman, Demming, 1987; Johanson et al., 1993). In the case of all three indicator species for old forests studied here, both immediately after closing the foliage canopy of trees and a month later, the values of this parameter were above 0.80 – the highest for *S. holostea* (Tab. 4).

Lower values of this parameter indicate increased plant stress (Maxwell, Johnson, 2000). A decrease in the Fv/Fm value indicates that less products of the photosynthetic light reactions are produced, and the value of this indicator also depends on the amount of photosynthetically active pigments (Porcar-Castell et al., 2008a, b). This may explain the slightly higher result of this parameter obtained here for *S. holostea*.

At the same time, parameters such as non-photochemical quenching (NPQ), i.e. dissipation of excess energy in the form of heat, reached higher values immediately after closure of tree leaf canopy, in the first term for *G. luteum* and *A. nemorosa* (Tab. 5). For example, in the corn experiment, the lowest NPQ values were recorded at low intensity of photosynthetically active radiation (Drożak, Romanowska, 2006). According to some researchers, the increase in NPQ may be the result of increased heat energy removal (Heber et al., 2006), which would not be a strange phenomenon under the open forest canopy conditions. The PSII vitality index (Rfd) in *G. luteum* slightly increased at the second date, and decreased in *A. nemorosa* (Tab. 5). The decrease in the value of this indicator may in the latter case be caused by the preparation for the resting stage of this plant. This can also be confirmed by the intensively occurring biochemical processes at that time, which is indicated by the higher value of stationary fluorescence (Ft), illustrating the amount of energy directed to carry out biochemical processes (Fracheboud et al., 2009).

Changes in fluorescence emission or its ratio (e.g. blue/red) can be indicators of plant stress or estimation of chlorophyll content (Buschmann, Lichtenthaler, 1998). In studies of indicator species of old forests, the shape of fluorescence emission spectra in the blue range, both in the first and second measurement dates, was similar (Fig. 4). Higher values for the analysed species were found just after the closure of the tree leaf canopy, in the first term. Blue-green fluorescence (F450–F530) is due to the presence of phenolic compounds in the leaf epidermis (e.g. Lang et al., 1991; Saja et al., 2016), which are produced under stress. The production of phenolic compounds is probably a sign of developing stress resistance (Randi et al., 2014). Perhaps there were some stress factors at work here. The emission of leaf fluorescence in the red range also had a similar spectral shape in all species. For *G. luteum*, higher values of these parameters were recorded in the first term, and for *A. nemorosa* one month after closure of tree leaf canopy. The source of fluorescence emission in this range is chlorophyll, constituting PSII reaction centres and antenna complexes (Gitelson et al., 1998). In the case of *A. nemorosa*, this is not fully reflected in the amount of chlorophyll, which was lower in July than in June (Fig. 3).

The most sensitive indicators showing the effect of a stressor on plant tissue are the fluorescence ratios of blue to red (F440/F695) and blue to far red (F440/F735) (Schweiger et al., 1996). A decrease in both F440/F695 and F440/F735 was found on the second measurement date only in the leaves of *G. luteum* (Tab. 6). The intensity

of blue and green fluorescence from plant leaves usually remains constant when the leaves are exposed to light (Stober, Lichtenthaler, 1993), in contrast to the red and far red fluorescence of chlorophyll (Richter, Lichtenthaler, 1996). In the leaves of *G. luteum* and *A. nemorosa*, the value of the F440/F595 coefficient was lower in the first measurement period. In the case of the PSI/PSII ratio, its lower values were recorded in the first period of harvest only in the leaves of *S. holostea*. According to Jena et al. (2012), under stress conditions, together with a decrease in the fluorescence intensity of both the antenna and cortical parts in PS, there is an increase in the ratio of their fluorescence intensity. The value of blue-green and red fluorescence intensity depends on the plant species and stress conditions in which it is located.

The research carried out here shows that indicator species of old forests, despite the high human pressure to which they are exposed in the isolated urban forests, are able to cope well enough to function normally. Many of their physiological reactions may be the result of natural phenological transformations, which are a consequence of changes in lighting, resulting from the structure of the forest, including the degree of overlying foliage density. Environmental stress factors may also add to this, but so far it is not so visible among the species analysed here. These phenomena are very interesting and not yet fully understood. Further studies of these indicator species of old forests are necessary to preserve the biodiversity of forests, more or less transformed by man (Dzwonko, Loster 1989; 2001), and learning about their physiology may contribute to developing more effective ways to protect them. Peterken (1974) was the first to point this out when he wrote about the importance of such indicators for nature conservation. Their occurrence can be a measure of the natural quality and value of the forest.

Summary and conclusion

The following detailed conclusions can be drawn from the studies carried out here: [1] The fresh mass of individual leaves of *Galeobdolon luteum* and *Anemone nemorosa* was significantly higher immediately after closing the foliage canopy of trees; one month later, *A. nemorosa* showed a decrease in leaf dry mass, and *S. holostea* showed a significant decrease in leaf water content; [2] In the leaves of *G. luteum* and *A. nemorosa*, a higher concentration of chlorophyll was found in the first analysis period, and in *S. holostea* one month after closing the foliage canopy of trees; these results were similar in both methods of testing these dyes; [3] In the case of maximum (F_m) and variable (F_v) fluorescence, a significant increase in the values of these parameters was noted in *S. holostea* in the second measurement date; a higher value of F_v/F_m was also found in *S. holostea* in the second period of measurements; for all species in both measurement dates the F_v/F_m ratio was higher than 0.80; [4] Non-photochemical quenching (NPQ) in leaves of *G. luteum* and *A. nemorosa* was higher immediately after closing the foliage

canopy of trees; the PSII vitality index (Rfd) in *G. luteum* slightly increased at the second date and decreased in *A. nemorosa*. In *S. holostea*, the NPQ and Rfd values were similar in both measurement dates; [5] The shape of the fluorescence emission spectra in the blue range, both in the first and second measurement dates, was similar and had two peaks – at a wavelength of about 530 nm and 590 nm; the red fluorescence emission of leaves also had a similar spectral shape in all species, with peaks at 690 nm and 735 nm.

Indicator species of old forests found in urban forests, despite anthropogenic pressure, are able to cope well enough to carry out their life processes normally, as evidenced by a well-functioning photosynthetic apparatus. Their physiological reactions may therefore be the result of natural phenological transformations, which are a consequence of changes in lighting, resulting from the structure of the forest. The research carried out here should be treated as a preliminary study for the broadly understood exploration of indicator species in disturbed forests with varying degrees of anthropogenic pressure. Only comparisons of the physiology of different, more or less disturbed and isolated complexes will show a greater detail of the physiological responses to environmental stress of these species.

Conflict of interest

The authors declare no conflict of interest related to this article.

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Abstract

The article concerns the study of selected physiological features of three indicator species of the undergrowth of the oak-hornbeam forest (*Anemone nemorosa* L., *Galeobdolon luteum* Huds. emend. Holub and *Stellaria holostea* L.), after the forest canopy closure. Las Wolski, an isolated forest complex located within the city of Cracow (Southern Poland), was selected for the study. The plant material was collected at two points in the season – right after the forest canopy closure and a month later. Under laboratory conditions, the physiological characteristics of the indicator species were analysed. Light stress may play a smaller role here, and physiological parameters are probably most affected by the phenology of undergrowth species, which is adapted to seasonal changes in habitat conditions. However, these issues require more research.

Keywords: *Anemone nemorosa*, anthropogenic pressure, biomass, chlorophyll, environmental stress, *Galeobdolon luteum*, phenology, PSII, *Stellaria holostea*

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Zmiany cech fizjologicznych gatunków wskaźnikowych runa starych lasów

Streszczenie

Opracowanie dotyczy badań wybranych cech fizjologicznych trzech gatunków wskaźnikowych runa lasu grądowego (*Anemone nemorosa* L., *Galeobdolon luteum* Huds. emend. Holub i *Stellaria holostea* L), po zwarciu okapu drzewostanu. Do badań wybrano Las Wolski, izolowany kompleks leśny, znajdujący się w obrębie miasta Krakowa (Południowa Polska). Materiał roślinny pobrano w dwóch punktach sezonu – zaraz po zwarciu okapu drzew i miesiąc później. W warunkach laboratoryjnych przenalizowano cechy fizjologiczne gatunków wskaźnikowych (biomasę liści, zawartość wody, zawartość chlorofilu, fluorescencję chlorofilu, wypływ elektrolitów z komórek liści). Badania pokazały, że stres świetlny może tu odgrywać mniejszą rolę, a na parametry fizjologiczne prawdopodobnie najważniejszy wpływ ma fenologia gatunków runa, która jest dostosowana do sezonowych zmian warunków oświetlenia. Gatunki wskaźnikowe starych lasów spotykane w lasach miejskich, pomimo antropopresji, potrafią sobie radzić na tyle dobrze, aby normalnie funkcjonować. Przeprowadzone tu badania należy traktować jako wstępne studium do eksploracji gatunków wskaźnikowych w lasach zaburzonych, o różnym stopniu antropopresji. Dopiero porównania fizjologii z różnych, mniej lub bardziej zaburzonych i izolowanych kompleksów, pokażą większą szczegółowość reakcji fizjologicznych na stres środowiskowy tych gatunków.

Słowa kluczowe: *Anemone nemorosa*, antropopresja, biomasa, chlorofil, stres środowiskowy, *Galeobdolon luteum*, fenologia, PSII, *Stellaria holostea*

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