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The Effect of Seasonality of Climate Conditions on the Structure and Functional Performance of Photosynthetic Apparatus of Medicinal Plant Petasites hybridus

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Abstract. The aim of this study was to relate annual variation in the structure and the functional performance of photosynthetic apparatus of a local population of medicinal plant *Petasites hybridus* (L.) to environmentally induced constraints in its natural habitat. The comparative analyses of chlorophyll thermoluminescence (TL) and photosynthetic oxygen evolution, used to assess functionality and recombination events of PSII reaction centres revealed specific changes in correlation to the climate conditions during the seasons. The analysis of lipid classes and fatty acids composition of monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyl diacylglycerol (SQDG) and phosphatidyldiacylglycerol (PG) show the existence of qualitative and quantitative differences that can contribute in this regard. The obtained results are discussed in terms of a possible relationship between seasonal variation of photosynthetic performance and modulation in the profile and accumulation of secondary metabolites with therapeutic activity in this medicinal plant.

Key words: *Petasites hybridus* L., chloroplasts, fatty acids, lipid composition, oxygen evolution, photosynthetic activity, thermoluminescence.

Introduction

The utilization of medicinal plants to produce natural compounds with important therapeutic properties has gained increasing attention over past decades due to growing tendency to replace synthetic drugs with natural ones (Jimenez-Garcia et al., 2013). The beneficial medicinal effects of plant materials typically result from the combinations of biologically active substances (BAS), products of secondary plant metabolism (Briskin, 2000). Much of the medical plants used in pharmacy and medicine are collected from their natural habitat. Fluctuations in environmental factors as well as various unfavorable situations known to influence

general metabolism should also impact on secondary metabolism, i.e., on the biosynthesis and accumulation of natural products (Selmar & Kleinwaechter, 2013). Clarification of these relationships is essential to define their impact on the efficacy and therapeutic potential of phytochemical preparations of medicinal plants (Gobbo-Neto & Lopes, 2007). In order to answer these questions in the present study, comparative analyses of the structure and functional activity of the photosynthetic apparatus of *Petasites hybridus* are provided depending on the seasonal variations in the climatic factors of the environment.

The genus *Petasites* Mill. (Asteraceae) is widely vegetated in Europe, Northwest Asia and North America and has a long history of use in alternative medicine. Taxonomic survey of *Petasites* reveals the existence of 18 species of this genus. In Bulgaria, three *Petasites* taxa can be naturally found: *P. hybridus* (L.) P. Gaertn., B. Mey. & Scherb. (= *P. officinalis* Moench), *P. albus* (L.) Gaertn. and *P. kablikianus* Tausch ex Bercht. Representatives of genus *Petasites* are shown to be potential sources of high levels of bioactive substances with very promising aspects of therapeutic utility (Aydın et al., 2012). Main biologically active compounds are secondary metabolites classified as sesquiterpene esters of petasin and isopetasin. Purified preparations of *P. hybridus* roots, free of pyrrolizidine alkaloids are finding an increasingly widespread pharmacological application for the prophylaxis and treatment of migraine, bronchial asthma and allergic rhinitis - diseases affecting many people (Lipton et al., 2004). Extracts are also successfully used for the prevention of gastric ulcer, urinary tract irritation and respiratory problems (Ziolo & Samochowiec, 1998). Comparing the results of the structural-functional peculiarities of photosynthetic apparatus during the seasons with planned detailed phytochemical analyses of the composition, amount and antioxidant capacity of secondary metabolites will contribute to elucidating the physiological basis of variation in the metabolic profile and the accumulation of biologically active substances of this medicinal plant.

Materials and Methods

The study was conducted with plants growing at the region of Kokalyane, Devil's bridge (lat. 42°33′37′ N, long. 23°25′18′ E; altitude 650 m; Vitosha Mountains region, humid continental climate). The measurements were done with isolated thylakoids from leaves collected on three occasions during 2019 (April, August and November), chosen to represent different temperature and water supply during the seasons.

Averaged samples of leaves of 3-4 plants collected during the respective seasons were homogenised in 50 mM Na-tricine (pH 7.8), containing 3 mM Na-ascorbate, 10 mM NaCl, 5 mM $MgCl_2$, 0.4 M sucrose and 5% PEG-6000. The resulting slurries were passed through 8 layers of cheesecloth and the broken chloroplasts (thylakoids) were collected by centrifugation at 1000 × g for 10 min. The pellets were washed twice in 10 mM Na-tricine (pH 7.8) containing 0.4 M sucrose, 10 mM NaCl and 5 mM MgCl and then resuspended to concentration of 1 mg Chl/ml in 50 mM Na-Mes (pH 6.5) instead of Tricine buffer and stored on ice for 1 h in the dark before measurements. The pigment content was determined spectrophotometrically (Lichtenthaler, 1987).

Thermoluminescence (TL) measurements were carried out in darkness using computerized equipment, described in detail in (Zeinalov & Maslenkova, 1996). Samples of isolated thylakoid membranes were illuminated at 2-5°C to generate charge pairs within the PSII reaction centres and then rapidly cooled down in liquid nitrogen to trap those charge-separated states. Subsequent warming of the samples reveals thermoluminescence emission with characteristic peaks (Sane & Rutherford, 1986). Decomposition an alysis of TL glow curves was carried out using Origin Pro 8.

Oxygen-evolving reactions were measured using polarographic oxygen rate electrode (Joliot-type) and thylakoid membranes (100 µl sample volume, 300 µgChl/ml) without any artificial electron acceptors, as described in (Zeinalov, 2002). Oxygen flash yields were induced by saturating (4 J) and short ($t_{1/2}$ = 10 µs) periodic flash sequences with 650 ms dark spacing between the flashes. The initial oxygen burst was recorded after irradiation with continuous white light (450 µmol photons m^{-2} s^{-1}). The kinetic parameters of oxygen evolution (initial S_o and S_1 state distribution, misses and double hits) were determined by the least square deviations fitting of the experimentally obtained oxygen flash yields with the theoretically calculated yields, according to the model of Kok et al. (1970). Deconvolution of the oxygen burst decay was performed by fitting of the function with two exponential components: $A_1e(tk_1) + A_2e(tk_2)$, where A_1 and A_2, and k_1 and k_2 were the rate constants of the fast and slow components of the oxygen burst decay, respectively.

For TEM analysis small segments (1-2 mm^2) from the middle part of fully expanded leaves were taken and fixed in 3% (m/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 12 h at 4 °C The leaf segments were post fixed in 1% (m/v) $KMnO_4$ in the same buffer for 2 h at room temperature. After dehydration by increasing concentrations of ethyl alcohol (from 25 to 100%), the samples were embedded in Durcupan (Fluka, Buchs, Switzerland) and cross-sectioned with Reichert-Jung (Wien, Austria) ultramicrotome. Observation and documentation were performed by JEOL 1200 EX (Tokyo, Japan) electron microscope.

The lipids were extracted with chloroform/methanol/water as described by Bligh & Dyer (1959). The fresh aerial part of plant (25-30 g) was homogenized with 20 ml methanol and refluxed for 5 minutes in order to inactivate the lipases. An equal volume of chloroform was added and after 24h the mixture was filtered, and an equal amount of water was added. The lower layer (total lipophilic extract) was evaporated under vacuum and kept at –30°C. The amounts of lipophilic components were determined gravimetrically.

For lipid classes and fatty acids analyses part of the total lipophilic extract (50 mg) was applied on 20 × 20 cm silica gel G (Merck) plate (layer thickness 0.5 mm) and then the plate was developed with chloroform-methanol-acetone-acetic acid (70:14:24:0.4 v/v/v/v) as a mobile phase. The spots of the main lipid classes were visualized under UV-light, scrapped off with the silica gel layer and transferred in small vials with Teflon screw caps. Five ml of 15% acetyl chloride in absolute methanol were added and the vials were heated for 4 hours at 55°C (Christie, 1989). After cooling, the samples were diluted with water and the obtained fatty acids methyl esters (FAME) were extracted twice with hexane (2 × 5 ml). The FAME in combined hexane extracts were purified by preparative thin–layer chromatography (TLC) on 20 × 20 cm silica gel G (Merck) plates (layer thickness 0.5 mm) developed with hexane–acetone (95:5 v/v). The spots of the FAME were visualized under UV light, scrapped off with the silica-gel layer and eluted with diethyl ether. The amount of each sample was determined gravimetrically.

Fatty acid analysis was performed using Gas Chromatograph with Flame Ionization Detector Agilent 7890B, equipped with Agilent7693 Autosampler with 10 µl syringe and with capillary column SGE BPX70 (60 m x 0.25 mm x 0.25 µm). Nitrogen was the carrier gas at flow rate of 1.2 ml/min. The column temperature was programmed from 80°C (hold for 1 min) to 130°C by step of 8°C/min and then to 250°C by step of 5°C/min. The injector and detector temperatures were 245°C and 255°C, respectively; split 15:1. Instrument control, data acquisition and data processing were performed by GC software Clarity v.8.0.0.125. The fatty acids were identified by comparison of the retention times with that of reference mixture F.A.M.E. Mix C8-C24

(Sigma-Aldrich). The relative amounts of the fatty acids were determined from peak areas of the respective methyl esters.

The fluidity of the membrane lipids was expressed by the level of unsaturation, calculated as unsaturated/saturated ratio (16:1+18:1+18:2+18:3)/ (16:0+18:0).

Results and Discussion

The production of secondary metabolites by plants growing in natural populations is conditioned by environmental factors (Gobbo-Neto & Lopes, 2007). Constantly changing environmental conditions (temperature, water supply, light intensity, UV radiation, etc.) induce physiological adaptation by influencing the activity of primary metabolic reactions such as photosynthesis, which is a major physiological process that determines plant growth and productivity. Properties of photosynthetic apparatus may contribute to a great extent to plant habitat separation and adaptation to environmental factors, but currently there are no comprehensive studies on the effects of abiotic and phenological factors on growth, photosynthetic activity and accumulation of sesquiterpenes in *Petasites.* The most common species and the main medicinal plant in *Petasites* genus, used in European phototherapy, is *P. hybridus* (common butterbur). The plant generally inhabits humid areas preferably in partial shade, but if there is appropriate amount of water, it can grow in full sun. Plants are considered drought intolerant compared with many other plant species and they can encounter temporal increases in drought stress during summer due to increasing temperatures, decreasing precipitation, or both. *Petasites* blooms in the spring, and later develops leaves, thus early flowering may be considered as an advantageous defense strategy of the plant. The environmental conditions during the measurement periods were characterized by average spring (in April) and autumn (in November) minimum and maximum temperatures of 5 and 16.7°C, and 6.2 and 13.7°C, respectively, and there was non-limiting water supply (75.7 mm and 69.7 mm average monthly amount of precipitation). For the summer-grown plants (in August) the corresponding temperature values were 15.4 and 30.1°C and there was a progressive increase in drought from spring to summer (34 mm average monthly amount of precipitation). The analysis of plant water status of investigated samples determined by measuring the hydration of leaves (H[gH_2O(gDW) show lower values of summer (3.66) and autumn (4.5) in comparison to spring samples (5.75). Similar values were obtained in preliminary studies in two-year period (2015-2016, unpublished data). Drought stress (i.e. the combination of water deficit, high light and high temperatures) can reduce stomatal conductance, making the photosynthetic apparatus susceptible to photodamage. Perturbations by stressful environments are first manifested in alterations in structure of thylakoid membranes and the photochemical efficiency of photosystems, especially photosystem II (PSII). Moreover, PSII oxygen-evolving enzyme complex of thylakoid membranes appears main stress sensitive site in plants. The possibility of fast and reliable monitoring of the effectiveness of the operation of PSII centers is the first prerequisite to solve site and mechanisms of stress injury and adaptation to biotic and abiotic environmental factors. In this regard, the kinetic analysis of oxygen-evolving reactions in continuous and pulsed lighting and thermoluminescence (TL) emission as indicators of energy balance and functioning of PSII reaction centers, have important application in plant stress physiology.

Upon illumination by a single turn-over flash the isolated chloroplasts showed a TL emission curve which could be well fitted with only one component representing the main TL B-band ($S_{2(3)}Q_B^-$ charge recombination), related to dark distribution of the S_2 and S_3-states of oxygen evolving complex and Q_B/Q_B^- ratio. The analysis of the obtained data (Table 1) revealed distinct differences in TL parameters. In summer

samples the relative contributions of B-band to the overall TL emission were significantly lower (29.6%) in comparison to spring samples (44.7%) and a shift of the maximal emission temperature of B band from 27.5°C to lover values (23.5) was also observed. During autumn, the relative contribution of B-band increases to 35.7% accompanied by T_{max}, centered at 25°C. It is reasonable to suggest that the TL data reflects seasonal dynamics in the relative number and stability of PSII reaction centers.

Another reliable approach used to study the properties of PSII complex in *Petasites* thylakoids in correlation to the climate conditions during the seasons was analysis of the kinetics of oxygen-evolving reactions under continuous and flash excitation (Maslenkova et al., 1993). The amplitudes of the oxygen burst A (%) under continuous irradiation, which is a measure of the oxygen volume evolved, differ in thylakoids from the investigated samples, reaching the lowest values in summer thylakoids (Table 1). The induction curves after oxygen burst exhibit biphasic exponential decay. The ratio between the amplitudes of the fast (A_1) and the slow (A_2) components after decomposition of oxygen induction curves and the respective time constants t_1 and t_2 (Table 1) suggest a decrease in the proportion of functionally active PSIIα centers in thylakoids from summer plants which could be attributed to the reduced grana formation and dominant operation of the cooperative mechanism of oxygen evolution in stroma situated PSIIβ centers. It is supposed that the cooperative mechanism is realized by diffusion of oxygen precursors mainly within PSIIβ centers and is characterized by a time constant lower than that of the non-cooperative Kok's mechanism, realized by PSIIα centers (Zeinalov & Maslenkova, 1996). Analysis of the flash-induced oxygen yield patterns reveals significant inhibition of the active PSII centers (S_0+$S_{1)}$ that evolve oxygen by non-cooperative mechanism (PSIIα centers) for summer samples without changes in miss parameter, suggesting that the resting PSII centers functioned nearly as in the spring plants, accepted as control. A gradual increase in the number of the active PSII centers (to 51.7%) during autumn is observed.

T(ºC) and B (%) are B band emission temperature, recorded after one turn-over flash and the contribution of B-band to overall TL emission; A is the amplitude of the initial oxygen burst; A_1 and A_2, and t_1 and t_2 represent amplitudes and time constants of fast and slow components of initial oxygen burst; S_0+S_1 and α and β are the sum of active oxygen evolving centers working through non-cooperative mechanism; α and β are the values of misses and double hits, according to the Kok's model.

The presented comparative analysis of the functional properties of the PSII complex during the seasons show an increased proportion of inactive (photoinhibited) PSII centers during summer as precipitation decreases and temperature rises, i.e. when drought increases and gradually recovered as temperatures fall and appropriate water supply are established during the autumn (Table 1). The number and the stability of PSII centers were maximal in spring growth conditions when water was abundant and temperature was moderately high. The observed lover values in PSII activity of autumn are most likely due to aging processes of chloroplasts (Fig. 1c).

To the best of our knowledge there are no microscopic investigations on chloroplast structure in *Petasites* leaves. The data obtained by TEM-analysis revealed that the structure of the chloroplasts in leaves collected in spring were characterized by an elliptical shape and well-developed inner membrane system (Fig. 1a). The grana represented different height. The number of thylakoids in them varies from 8-10 to 30, connected by well-developed and evenly spaced stromal thylakoids.

The inner membrane system of summer chloroplasts was characterized by an increase in the number of lower grana with a smaller number of thylakoids (8-15) and stromal

thylakoids of greater length. A few small plastoglobulus in the stroma were noted. The chloroplasts of autumn collected leaves differed from the spring chloroplasts by the great amount of plastoglobulus and relatively small proportion of the internal membrane system, which were related to the natural aging processes.

Table 1. Seasonal changes in the kinetic parameters of thermoluminescence (TL) and oxygen evolution in isolated thylakoids.

Sample	T (°C)	B (%)	A (%)	A_1/A_2	t_1 [s]	t_2 [s]	S_0+S_1 (%)	α (%)	β (%)
Spring	27.5	44.7	100.0	2.09	0.32	2.78	100.0	15.6	4.9
Summer	23.5	29.6	28.6	0.56	0.57	4.1	20.3	16.1	4.5
Autumn	25.0	35.7	61.4	1.38	0.44	3.6	51.7	15.2	5.1

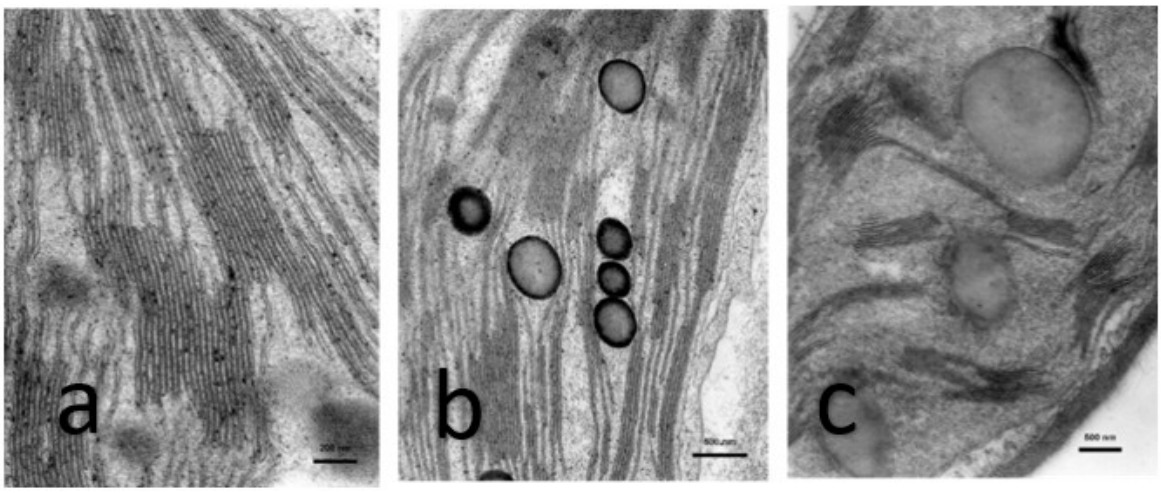

Fig. 1. Chloroplast ultrastructure in **a**: spring, **b**: summer, **c**: autumn.

It is well known that lipids play an essential role in maintaining the integrity and functional activity of chloroplast membrane. In this respect the elucidation the impact of varying environmental conditions during seasons and phenological factors on changes in lipid classes and fatty acid composition of *Petasites* membranes could bring important information in the interpretation of the obtained results. The lipid classes of thylakoid membranes were quantified at three different months during spring, summer and autumn (Table 2).

Photosynthetic membranes of plants are characterized by a high content of glycolipids dominated by galactolipids MGDG and DGDG. The fraction of MGDG slightly increase in summer, but the MGDG/DGDG ratio (1.1) is equal to the spring samples (Table 2). During the autumn fractions of MGDG in the membrane decreased and the MGDG/DGDG ratio also decreased (to 0.69) at the expense of the increased amount of DGDG. The fractions of the minor lipid constituents of thylakoid membrane show a gradual decrease of the amount of SQDG from spring to autumn while the amount of PG was found nearly constant during the seasons.

Six common fatty acids were detected in the lipid classes of the respective thylakoid

preparations, including palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) and the seasonal variations in their relative content are presented in Table 3. Small quantities of some unusual fatty acids, early reported for *Petasites* thylakoid preparations (Yordanova et al., 2017) are not included in this analysis. The most abundant fatty acid in all the lipid classes was linolenic acid followed by palmitic and linoleic acids. The results revealed changes in fatty acids profiles of MGDG and DGDG in summer chloroplast membranes including decreased level in trienoic fatty acids and consistent increases in saturated 16:0 and 18:0 and in dienoic 18:2 levels. The changes in the level of unsaturation (Table 3), calculated as unsaturated/saturated ratio (16:1+18:1+18:2+18:3)/(16:0+18:0) were most obvious for MGDG reaching lower values during the summer and increasing during spring and autumn. The observed changes are consistent with the data for PSII activity of studied samples (Table 1). The content of polyunsaturated fatty acids in the lipid matrix is one of the major factors determining membrane fluidity. The decreased level of lipids unsaturation reduces the mobility of lateral separated pigment-protein complexes and electron carriers in the electron transport chain thus affecting the effectiveness of photosynthetic machinery. It has been proven that polyunsaturated fatty acids in thylakoid lipids play an important role in the stability of oxygen evolving machinery and increases in unsaturated fatty acids in membrane lipids protects PSII against photoinhibition (Sui & Han, 2014).

Table 2. Lipid classes in thylakoid membranes. The values obtained are means ± s.e. from three parallel measurements.

Seasons	Lipid classes (% of total)			
	MGDG	DGDG	SQDG	PG
Spring	25.92±1.1	25.92±1.0	27.77±0.7	20.37±1.0
Summer	30.76±1.0	27.70±0.4	23.07±0.5	18.46±0.9
Autumn	22.92±0.8	33.33±0.8	20.83±1.2	22.92±0.5

Table 3. Seasonal variations in relative fatty acids content of lipid classes. The values obtained are means ± s.e. from three parallel measurements; U/S, unsaturated to saturated fatty acids ratio; n.d. – not detected.

Membrane lipids	Sample	16:0	16:1	18:0	18:1	18:2	18:3	U/S
MGDG	spring	5.6±0.3	n.d.	1.4±0.2	1.7±0.7	1.6±0.2	88.2±0.1	12.96
	summer	7.9±1.2	n.d.	1.5±0.3	3.3±0.8	3.5±1.3	79.9±0.2	9.24
	autumn	2.5±0.6	n.d.	0.8±0.9	1.8±0.4	1.3±0.3	87.4±2.2	22.07
DGDG	spring	17.9±0.3	n.d.	2.8±0.3	n.d.	2.6±0.4	74.4±0.8	3.72
	summer	17.4±0.5	0.6±01	3.4±0.6	2.0±0.2	3.1±0.7	68.5±0.1	3.54
	autumn	12.4±1.1	0.5±0.1	2.7±0.1	1.9±0.4	2.0±0.2	75.3±1.4	5.05
SQDG	spring	27.6±2.3	9.9±0.7	4.2±0.8	1.9±1.3	9.6±1.8	38.5±0.1	1.98
	summer	27.7±0.6	13.0±0.4	2.3±0.3	1.9±1.1	11.1±0.1	38.9±1.7	2.03
	autumn	24.9±0.7	1.2±0.7	3.3±0.3	2.6±0.7	5.4±0.8	48.1±1.3	2.03
PG	spring	27.2±0.3	6.6±1.2	5.0±0.1	1.1±0.4	6.6±0.1	30.9±0.1	1.38
	summer	24.2±1.0	4.4±0.7	3.1±0.4	6.6±0.5	11.7±0.1	31.2±0.5	1.59
	autumn	28.6±1.1	10.9±0.2	3.0±0.6	2.9±1.2	6.5±0.1	37.2±0.2	1.82

Conclusion

The results of the present study on a local populatiobn of *Petasites hybridus* plants show seasonal variations in the activity of the photosynthetic apparatus expressed in strong inhibition of PSII activity during summer followed by a process of recovery during autumn and reacing maximal values in spring. These changes in photosynthetic performance are accompanied by modifications in the structute and composition of chloroplast membranes, connected with changes in membrane lipids and their fatty acid composition. The amount of unsaturated fatty acids is consistent with the data for PSII activity of studied species. A low level of unsaturation in summer thylakoid membranes makes PSII of drought intolerant *Petasite* extremely susceptible to photoinhibition and causes a significant reduction in photosynthetic activity. Unfavourable environmental conditions during summer induces stress-related metabolic responses as a result of which metabolic processes are shifted towards biosynthetic activities that consume reduction equivalents, especially the synthesis of secondary compounds such as terpenoids, phenols or alkaloids. Medicinal plants grown under water deficiency conditions reveal much higher concentrations of relevant natural products.

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