Relations between Photosynthetic Performance and Polyphenolics Productivity of *Artemisia alba* Turra in *in vitro* Tissue Cultures

Nia Petrova[1], Petya Koleva[2], Violeta Velikova[3], Tsonko Tsonev[3], Tonya Andreeva[1], Stefka Taneva[1], Sashka Krumova[1], Kalina Danova[2*]

[1]*Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences*
Acad. Georgi Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria
E-mails: zlatkova.nia@gmail.com, t_andreeva@abv.bg, sgtaneva@gmail.com, sashka@bio21.bas.bg

[2]*Institute of Organic Chemistry with Centre of Phytochemistry*
Bulgarian Academy of Sciences
Acad. G. Bontchev Str., Bl. 9, 1113 Sofia, Bulgaria
E-mails: danova@abv.bg, petya_k0leva@abv.bg

[3]*Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences*
Acad. Georgi Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria
E-mails: violet@bio21.bas.bg, tsonev@gmail.com

[*]*Corresponding author*

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Abstract: *Establishing optimal growth conditions for secondary metabolites production in vitro is vital for the biotechnological development of medicinal plants. In the present work we investigate the relations between the supplementation of plant growth regulators (benzyl adenine and indole-3-butyric acid) to in vitro shoot cultures of the medicinal plant Artemisia alba Turra, the productivity of antioxidant polyphenolic compounds and the structural and functional characteristics of the photosynthetic apparatus. We assayed the structural characteristics of isolated thylakoid membranes from the aerial parts by means of circular dichroism spectroscopy and atomic force microscopy, and the photosynthetic performance by pulse amplitude fluorescence modulated imaging.*
Although a complex relationship between benzyl adenine and indole-3-butyric acid supplementation, the polyphenolic levels and the architecture and functionality of the photosynthetic thylakoid membranes was revealed, a clear correlation was established between the concentration of the produced polyphenolic compounds and the quantum yield of photosystem II. Our data demonstrate that there is an optimal combination of the applied plant growth regulators that triggers efficient photosynthesis and high phenolics production.

Keywords: *Photosynthesys, Artemisia alba Turra, Polyphenolic compounds, in vitro shoot cultures.*

Introduction

Ethnopharmacological data reveal that application of *Artemisia* genus species in both Western and Eastern traditional medicine dates back to ancient times. Since then different *Artemisia* species have been irreplaceable as spasmolytic, analgesic and sedative agents used in cases of diabetes, headaches, gastric, respiratory, nervous and other diseases [16]. In present days this fact has inspired intensive research of the phytochemicals produced by species of this genus. The discovery of *A. annua* derived artemisinin in 1970s by Dr. Youyou Tu culminated in 2015 in the Nobel Prize in Physiology or Medicine awarded to Dr. Tu "for her discoveries concerning a novel therapy against Malaria", a breakthrough that is estimated to save

approximately 100 000 lives in Africa alone [12]. The diversity of this genus (it is comprised of more than 500 species [24]) together with its capacity to produce phytochemicals possessing curative properties (sesquiterpenes, monoterpenes, tannins, phenolics, flavonoids, coumarins, etc., see [1] for a comprehensive review) make *Artemisia* species extremely promising as a potential source of precursors for innovative medical products.

Artemisia alba Turra, also known as *A. lobelii* All., *A. biasolettiana* Vis., *A. suavis* Jord., *A. incanescens* Jord., *A. camphorata* Vill., is an aromatic shrub that is widely spread in Southern Europe [23]. It produces a large variety of secondary chemical compounds including terpenes [22] and phenolics [11]. The production of these classes of secondary metabolites in different species have been shown to be strongly dependent on the environmental factors such as light quality, temperature [25], salinity [10], addition of elicitors and plant growth regulators [2] etc., and these dependencies are widely applied as a tool for manipulation of targeted phytochemicals production. Previously, it was shown that alterations in sugar, vitamins and growth regulators content of *in vitro* cultures of *A. alba* greatly influences essential oil composition [3, 13, 19] and phenolics content [11] of *A. alba* plants.

For obtaining sustainable secondary metabolites producing system it is vital to generate *in vitro* system that does not compromise plant vitality and that can be monitored regularly. In this work we aimed at developing a system for targeted phenolics and flavonoids production that also has optimal photosynthetic performance. Phenolics are a very vast group of structurally diverse secondary metabolites that underlay many processes of plant development and adaptation. Flavonoids represent a major class of phenolic compounds, very actively participating in plant's survival mechanisms such as scavenging of reactive oxygen species, preserving the structural integrity of cell membranes, protecting plant's photosynthetic apparatus from excessive light intensity and UV light [17]. The photosynthetic reactions and complexes are a very reliable probe for plants fitness and knowledge on the relation between phenolics accumulation and photosynthesis is of interest since it is informative for both the plants physiological state, and their capacity for larger scale phenols production.

In the present work we determined a number of photosynthetic parameters for different lines of *in vitro* cultures of *A. alba* with known phenolics and flavonoids content obtained by treatments with two plant growth regulators (PGRs): cytokinine benzyladenine (BA) and auxin indole-3-butyric acid (IBA) [11]. We probed the plant photochemical efficiency and thylakoid membrane structural organization as reliable stress indicators and correlated the changes in *in vitro* plant's fitness with the level of the produced secondary metabolites.

Materials and methods

Cultivation conditions

A. alba tissue cultures were initialized from surface sterilized stem segments of field grown *A. alba* plants and cultivated at 25 ± 0.2 °C and 16/8 h photoperiod as in [3, 11]. PGR treatments were performed according to [11]. Intact *in vitro* grown plantlets were examined after 12 weeks of cultivation.

Thylakoid membranes preparation

Thylakoid membranes were isolated from dark-adapted 12-week-old *in vitro* grown *A. alba* plants. The isolation procedure was performed as described in [9].

Structural characterization of thylakoids

Circular dichroism (CD) measurements were performed by means of Jobin Yvon CD6 dichrograph. Spectra of thylakoid membranes were recorded between 400 and 750 nm with a scanning step of 1 nm and 0.2 sec integration time. Sample chlorophyll (chl) concentration was 15 μg/ml.

The topography of thylakoid membranes was probed by means of NanoScopeV, (Bruker Inc.) atomic force microscope (AFM) in a tapping mode, in air. Silicon cantilevers (Tap300Al-G, Budget Sensors, Innovative solutions Ltd., Bulgaria) with tip radius < 10 nm were used. The cantilever spring constant was 1.5-15 N/m and the resonance frequency was 150 ± 75 kHz. The images were scanned with a rate of 0.2 Hz with a resolution of 512 × 512 pixels. 2% (v/v) glutaraldehyde was added to the thylakoid membranes suspension (with a concentration of 5 μg chl/ml) prior to spreading on a freshly cleaved mica surface covered with 0.01% poly-L-lysine monolayer. After 20 min of incubation, the samples were washed with suspension buffer and dried with nitrogen flow. Thylakoids were approximated to ellipsoid with volume $V = 4/3\pi abh$, where a and b are the short and long axes of the ellipse and h is the height of the thylakoid.

Functional characterization of thylakoids

Functionality of photosynthetic apparatus of intact *A. alba* plants was studied by IMAGING-PAM MAXI version (Walz, Germany) supplied with LED-array-illumination unit (blue) IMAG-MAX/L for fluorescence excitation and actinic illumination with blue light as well as asseement of absorbed photosynthetically active radiation with the help of red light (650 nm) and NIR-light (780 nm), and CCD camera (IMAG-K7, 640 × 480 pixel). Plants were dark adapted for 40 min, and minimal fluorescence level F_o and maximal fluorescence level, F_m at dark adapted state were measured immediately after pulling the plants out of the culturing flasks, after that actinic light of 111 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 14 saturating light pulses in intervals of 30 seconds were applied until steady-state fluorescence, F_s was reached. The obtained images were processed by means of ImagingWinGiE V2.45i software. The photosynthetic parameters were evaluated for completely differentiated parts of the shoots.

Results and discussion

The flavonoid content of the studied GAIP variants followed similar pattern as the phenolics content (Table 1). IBA applied alone (GAIP_1 and GAIP_2) provoked a decrease in the mean total concentration of polyphenols (by 7-12%) and flavonoids (by 18-30%), while application of the cytokinine BA (alone or in combination with IBA) led to significant increase in phenolics and flavonoids accumulation (with exception of GAIP_8). The effect of increase of phenolics and flavonoids was most prominent for GAIP_4 (Table 1).

Many investigations show that plant phytohormones, and especially cytokinins, are among the most powerful factors that influence chl synthesis [6] and transcription of the major chl a/b binding light-harvesting protein [4, 7], these compounds also play a crucial role in preservation of the chloroplast ultrastructure [26]. In the light of these studies it can be expected that the overall structural organization of the chl containing photosynthetic protein complexes, as well as the whole thylakoid membrane, should be affected by treatment of *A. alba in vitro* cultures with BA and IBA. CD spectroscopy offers a non-invasive approach for such studies since it was shown that the intensity of the 696-675 nm CD band strongly depends on the relative size of the ordered chiral domains of photosystem II supercomplexes (formed in the granal segments of thylakoids) that have sizes of hundreds of nanometers and ensure strong excitonic coupling between the complexes. Our data show that the application

of IBA alone (GAIP_1 and GAIP_2) and low BA concentration – 0.2 mg/L (GAIP_5) does not change the intensity of this CD band as compared to the control GAIP_0 variant, while in all other variants on the average its intensity is reduced by 15-25% compared to GAIP_0 (Fig. 1A). This clearly demonstrates that the macroorganization of thylakoids strongly depends on the concentrations of the applied PGR in a non-linear manner. Also the combinations of IBA and BA, as well as high concentrations of BA result in reduction of the chiral domains in thylakoids. For GAIP_0, GAIP_1 and GAIP_2 the thylakoid macroorganization was not changed and the level of phenolics and flavonoids remained low in comparison to the other studied variants. On the other hand the reduction in the thylakoid membrane order was accompanied with a higher level of polyphenols and flavonoids as compared to the control GAIP_0 variant (with the exception of GAIP_8 which had low phenolics and flavonoids content but altered thylakoid membrane macroorganization).

Table 1. Treatments of *A. alba in vitro* tissue cultures with different concentrations and combinations of the plant growth regulators IBA and BA, and the respective total polyphenolic and flavonoids content (mean ± SE) of the different variants (source data [11])

***A. alba* variant**	**IBA (mg/L)**	**BA (mg/L)**	**Total phenolics (mg/g DW)**	**Total flavonoids (mg/g DW)**
GAIP 0	0	0	15.25 ± 0.31^{c}	3.48 ± 0.15^{c}
GAIP 1	0.5	0	13.49 ± 0.18^{d}	2.45 ± 0.49^{d}
GAIP 2	1.0	0	14.14 ± 0.94^{cd}	2.87 ± 0.22^{d}
GAIP 3	0.5	0.2	20.39 ± 0.52^{a}	4.51 ± 0.11
GAIP 4	1.0	0.2	22.32 ± 0.51	4.85 ± 0.20^{a}
GAIP 5	0	0.2	20.34 ± 0.56^{a}	4.74 ± 0.10^{a}
GAIP 6	0	0.7	16.38 ± 0.31	3.90 ± 0.06^{b}
GAIP 7	0.8	0.2	18.52 ± 0.47^{b}	4.17 ± 0.08
GAIP 8	0.5	0.7	14.58 ± 0.35^{c}	3.38 ± 0.04^{c}
GAIP 9	1	0.7	18.24 ± 0.59^{b}	3.92 ± 0.12^{b}

Material was collected of at least 15 individual plantlets, cultivated in 5 separate culture vessels. All measurements were performed in triplicate with three repetitions. Comparison of means was conducted by the Student t test for unequal variances. The differences were compared at $p \leq 0.05$. Same letters denote non-significant differences. Values designated with the same superscript (a, b, c, d) do not differ significantly.

So far, many authors have considered chloroplast structure as a significant factor in plant *in vitro* culture survival ([20] and references therein) and have related their secondary metabolites production potential to species specific alterations in chloroplast structural characteristics [14, 21]. In order to probe if the modified membrane macroorganization is associated with changes in the overall architecture of thylakoids we applied AFM. The mean values for the determined thylakoids volumes of the different PGR treated variants did not differ from those estimated for GAIP_0 (data not shown). However, closer inspection of the data revealed that for all variants there are at least two populations (fractions) of thylakoids with dramatically different volumes (Fig. 1B). Similarly to the CD data the variants with low polyphenols content (GAIP_0, GAIP_1 and GAIP_2) exhibit similar behaviour and had thylakoid fractions with comparable volumes. For GAIP_3 and GAIP_4 variants (that had maximal amount of polyphenols) we observed a small (17%) fraction of thylakoids with volume larger than 40 μm^3. For the rest of the variants (with intermediate level of polyphenols) the volume of the larger fraction was lower than the control GAIP_0.

The variants GAIP_6 and GAIP_7 showed the highest abundance of the larger thylakoid fraction (67-91%), while for the other variants it was 55% at most.

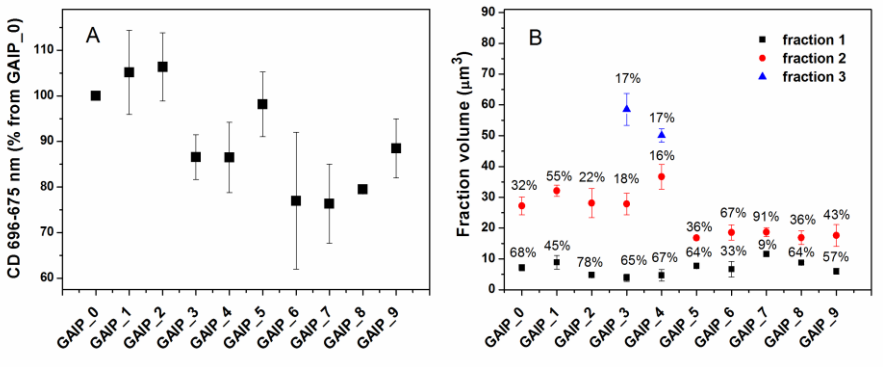

Fig. 1 Structural characteristics of *A. alba* thylakoid membranes:
(A) Intensity of the 696-675 nm CD band, relative to that of the respective control;
(B) Fractions of thylakoids with different volume; the relative abundance of each fraction (in % from the total number of studied objects for each GAIP variant) is denoted for clarity.

To test if the observed structural changes affect the functionality of the photosynthetic apparatus we used PAM imaging system that generates a color coded map of the fluorescent parameters of intact plants [15]. For the purpose of our study we investigated only the completely differentiated parts of the shoots. Our data showed that all treated variants are in a good functional state and no signs of inhibition were visible since all PGR treated samples had quantum yield of photochemical reactions of photosystem II in dark adapted state, F_v/F_m, similar to or higher than that of GAIP_0. The F_v/F_m value, the electron transport rate through photosystem II (ETR) and the level of non-photochemical quenching (NPQ), a measure of plants ability for photoprotection, for each of the studied GAIP variants are presented in Fig. 2A. Clear correspondence between the F_v/F_m values and the level of polyphenols (Fig. 2A) and flavonoids (data not shown) is observed – variants with high F_v/F_m values synthesized high amounts of polyphenols and flavonoids and vice versa, a moderate Pearson's correlation (R = 0.58) was estimated for those parameters. Different dependencies however were observed for the ETR and NPQ parameters. Variants GAIP_2-GAIP_6 exhibited higher values for ETR than the untreated GAIP_0 variant, while for GAIP_1, GAIP_7, GAIP_8 and GAIP_9 it remained close to the control (Fig. 2B). Variants GAIP_4 and GAIP_5 that had high polyphenolic and flavonoids content, also had the lowest NPQ; however NPQ did not change significantly for GAIP_3 that had similar content of polyphenolic and flavonoids (Fig. 2C).

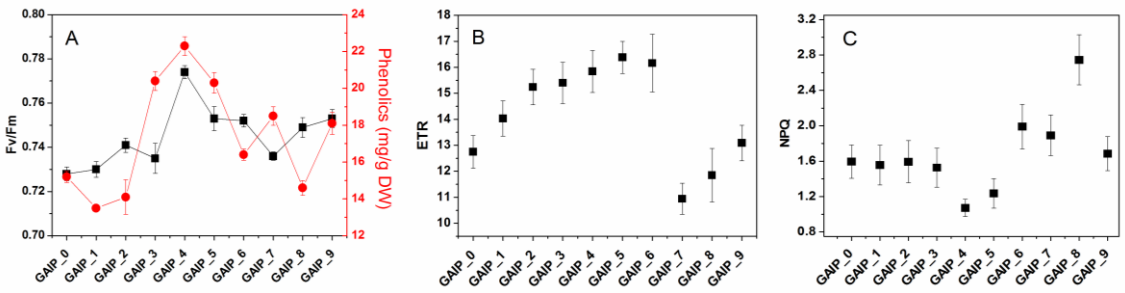

Fig. 2 Functional characteristics of *A. alba*:
(A) maximal photosystem II yield in dark-adapted state, F_v/F_m (■), where $F_v = F_m - F_o$. For comparison the concentration of phenolic compounds (•) is also plotted for each GAIP variant. (B) Electron transport rate, ETR.
(C) non-photochemical quenching, NPQ. All data are mean ± SE.

Among all studied PGR-treated variants, GAIP_4 strikes the attention as the one with the most efficient photosynthetic performance – highest F_v/F_m, very high ETR, lowest NPQ and the highest polyphenols and flavonoids content. This effect might be explained with the important function of polyphenols as excess light "filter" [8], as well as their ability to prevent overreduction of the electron transport chain in photosynthetic membranes [5, 8]. The presented results confirm the protective role of polyphenols for the function and structure of the photosynthetic apparatus subjected to variety of stress factors. Furthermore, it is found that the combination of 1.0 mg/L IBA and 0.2 mg/L BA (GAIP_4) is optimal for the functionality of *A. alba* and the production of polyphenols in tissue cultures from this shrub.

Our data reveal that the effects of exogenously applied PGRs on plants development are diverse and affect plastid development, thylakoid membranes macroorganization and the function of protein-pigment complexes comprising the photosynthetic apparatus, possibly via changes occurring in the phytohormone homeostasis of *A. alba* plants. This hypothesis is supported by the morphometric and endogenous cytokinin content data presented in [3, 11] which suggest that alterations in the root development (provoked by IBA and BA treatment) are the most possible reason for changes in phytohormone (cytokinin) homeostasis in *A. alba in vitro* shoot cultures. Mathematical approaches for the analysis of large databases combining data from different methods and for different metabolites concentrations might prove helpful to further analyze the complex molecular interaction networks between the various plant metabolites and their effects on the photosynthetic performace, as well as to optimize the cultivation conditions [18].

Conclusion

In this work we studied the relation between the supplementation of the growth medium with plant growth regulators (IBA and BA), the structural and functional characteristics of the photosynthetic apparatus and the total polyphenols and flavonoids content in *in vitro* tissue cultures of *A. alba*. We found that the structural features of thylakoid membranes correlate with the PGR, polyphenolics and flavonoids concentration, also a strong correspondence was established between the quantum yield of photochemical reactions of photosystem II in dark adapted state (F_v/F_m parameter), NPQ level and polyphenols and flavonoids accumulation. The applied approach helped to determine the optimal concentration and combination of IBA and BA that rendered both high photosynthetic efficiency and high accumulation of polyphenols and flavonoids.

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Nia Petrova, Ph.D. Student
E-mail: zlatkova.nia@gmail.com

Nia Petrova obtained a Bachelor degree in Molecular biology (2014) and a Master degree in Plant biotechnology (2015) at the Sofia University "St. Kliment Ohridski". Currently she is a Ph.D. student at the Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences (BAS). Besides her thesis topic which is related to the thermodynamic behavior of photosynthetic pigment-protein complexes of higher plants, she also actively takes part in projects in the field of cyanobacterial photosynthesis and medicinal plants in vitro cultivation.

Petya Koleva, M.Sc.
E-mail: petya_k0leva@abv.bg

Petya Koleva has obtained her Bachelor degree in Biotechnolgy and then Master degree in the subject of Cell Biology and Pathology at the Faculty of Biology, Sofia University "St. Kliment Ohridski". She was employed at the Institute of Organic Chemistry with centre of Phytochemistry, BAS, and has worked at the field of plant cell tissue and organ culture of medicinal and aromatic plants.

Prof. Violeta Velikova, Ph.D.
E-mail: violet@bio21.bas.bg

Violeta Velikova holds a Professor position at Institute of Plant Physiology and Genetics, BAS. She has 21-year professional experience in the field of plant ecophysiology. In the last 17 years, the focus of the studies is the interaction between biosphere and atmosphere with emphasis on emissions of biogenic volatile organic compounds, and on primary and secondary metabolism of plants under environmental constrains.

Prof. Tsonko Tsonev, Ph.D.
E-mail: tsonev@gmail.com

Tsonko Tsonev has worked in the Institute of Plant Physiology and Genetics, BAS. He has interests and experience related to the influence of unfavourable environmental factors (high and low temperatures, drought, heavy metals, photoinhibition, CO_2) on the functional activity of photosynthetic apparatus and its acclimation ability. Gas exchange and chlorophyll fluorescence as an informative criteria for the physiological state of photosynthetic apparatus are also objects of his research.

Assoc. Prof. Tonya Andreeva, Ph.D.
E-mail: t_andreeva@abv.bg

Tonya Andreeva is an Assoc. Prof. at the Institute of Biophysics and Biomedical Engineering, BAS. Dr. Andreeva has got her Ph.D. degree in Biophysics in the same Institute at 2006 with a thesis focused on the model lipid membranes with controllable morphology and surface potential. Until 2017 she published 28 research articles in the area of biophysics, plant science, physical chemistry, polymer science, biomedicine. She participated on more than 50 national and international conferences and in 20 scientific projects in collaboration with numerous national and international institutes and universities.

Prof. Stefka Taneva, D.Sc.
E-mail: sgtaneva@gmail.com

Stefka Taneva is a Professor at the Institute of Biophysics and Biomedical Engineering, BAS and a head of the Department of Biomacromolecules and Biomolecular Interactions. She graduated Physics and Biophysics at Sofia University "St. Kliment Ohridski". She received her Ph.D. degree in Physics from the Institute of Biophysics, Biological Research Center, Hungarian Academy of Sciences. The main focus of Prof. Taneva's research was thermodynamics of protein interactions, mechanisms of light-energy transduction – bacteriorhodopsin, macroorganization of pigment-protein complexes, static and dynamic electric properties of biological membranes; recent activity in application of microcalorimetry in disease diagnostics and in biofunctionalization of polyelectrolyte multilayers for medical application.

Assoc. Prof. Sashka Krumova, Ph.D.
E-mail: sashka@bio21.bas.bg

Sashka B. Krumova is an Assos. Prof. at the Institute of Biophysics and Biomedical Engineering, BAS. She graduated from Sofia University "St. Kliment Ohridski" in 2001 and obtained Ph.D. degree at the Institute of Biophysics, Sofia, Bulgaria and Wageningen University, The Netherlands. Her current research interests are in the fields of structural organization and thermodynamic properties of photosynthetic membranes, biomolecular interactions, biophysical approaches for disease diagnostics and monitoring.

Senior Assist. Prof. Kalina Danova, Ph.D.
E-mail: danova@abv.bg

Kalina Danova presently works at the Institute of Organic Chemistry with Centre of Phytochemistry, BAS. Her current research interests include plant cell tissue and organ culture of medicinal and aromatic plants for the production of secondary metabolites. Special focus is given to stimulation of their biosynthesis through optimization of growth conditions. Application of different organic/inorganic treatments is being applied and their effect on growth and developmental patterns is being studied.