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TERPENOID PROFILE OF *ARTEMISIA ALBA* IS RELATED TO ENDOGENOUS CYTOKININS *IN VITRO*

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Abstract

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Modifications of plant growth regulators supplied to *Artemisia alba in vitro* cultures have been previously shown to affect plant morphogenesis and terpenoid profile of the essential oil of aerial parts. We report here on the effects of plant growth regulators on the terpenoid profile, structure of photosystem II *in vitro* as well as on the endogenous cytokinin levels of both above- and underground parts. The contents of cytokinin bioactive forms (free bases and ribosides) were followed. It was revealed that the growth regulators-modified growth and development as well as the alterations of photosystem II structural organization were related to the cytokinin levels of *Artemisia alba*. Thus, elevated monoterpenoid levels were associated with a higher peripheral antennae aggregation and elevation of *trans*-zeatin riboside, dihydrozeatin and dihydrozeatin riboside as well as N^6-(2-isopentenyl) adenine in the aerials of the respective plant growth regulators treatments. These results imply of the role of exogenous factors such as cytokinins and auxins supplementation in affecting the terpenoid biogenesis *in vitro* by altering the levels of endogenous cytokinins and physiological status of the plant organism.

Key words: Artemisia alba, *in vitro,* endogenous cytokinins, Photosystem II, plant growth regulators, terpenoid profile
Abbreviations: PGR – plant growth regulators, CK – cytokinins, IBA – indole-3-butyric acid, BA – N^6-Benzyladenine, transZR – trans-zeatin riboside, DHZ – dihydrozeatin, DHZR – dihydrozeatin riboside, iP – N^6-(2-isopentenyl)adenine, transZ – trans-zeatin, DMAPP – dimethylallyl diphosphate

Introduction

Artemisia alba Turra is a fragrant shrub distributed in Southern Europe. Its essential oil possesses strong spasmolytic and antimicrobial properties (Ronse et al., 1990; Stojanovic et al., 2000). However, surveys have revealed a great variability of its terpenoid profile, attributed by different authors to environmental conditions, geographic distribution and/or to genetic factors (Radulović and Blagojević, 2010 and ref. cited within). Our previous research showed that stimulated root system development in *A. alba* tissue cultures caused by different plant growth regulator (PGR) treatments led to inhibited sesquiterpenoid formation and predominance of monoterpenoid components in the essential oil of the aerial parts. This raised a question of a possible root-to-shoot communication, which might possibly be responsible for the observed relations (Danova et al., 2012). As known, root apical meristems are considered the major sites of synthesis of the free cytokinins (CKs) in whole plants. The CKs synthesized in roots move through

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the xylem into the shoot, along with water and minerals taken up by the roots (Taiz and Zeiger, 2006). The CK levels in plants have been reported to be associated with different stages of plant development, the cell cycle, as well as with environmental factors such as mineral nutrients (especially nitrogenous) (Kakimoto, 2003 and references cited therein). This motivated us to search for possible relations between the terpenoid profile (both in the above- and underground parts), structure of the photosynthetic apparatus (as an indicator of the physiological status and adaptiveness of the plants to different media compositions) and the endogenous CK levels in *A. alba* as a result of exogenous PGR application.

Materials and Methods

***In vitro* cultures of *A. alba* –** shoot cultures were initiated from surface sterilized stem segments of the aerial parts of field grown *Artemisia alba* Turra plants as described previously (Danova et al., 2012). Five different culture media were used: the control medium without PGRs (GAIP_0) and the same containing 0.5 mg.L^{-1} IBA (GAIP_1); 1.0 mg.L^{-1} IBA (GAIP_2); 0.2 mg.L^{-1} BA + 0.5 mg.L^{-1} IBA (GAIP_3) and 0.2 mg.L^{-1} BA + 1.0 mg.L^{-1} IBA (GAIP_4). *A. alba* shoots were grown for 12 weeks at 25ºC and a 16/8 h photoperiod.

Essential oil preparation and GC and GC/MS analyses – the essential oil was prepared by micro steam distillation-extraction of the fresh shoots and underground parts (root/callus) of the *in vitro* grown plants in a modified Lickens-Nickerson apparatus for 2.5 h (Sandra and Bicchi, 1987) using diethyl ether as a solvent. GC analysis was performed under the experimental conditions reported earlier (Trendafilova et al., 2010). The individual components were identified by their retention indices (RI), referring to known compounds described in literature (Tkachev, 2008; Adams, 2009), and also by co-comparison of their MS, with those of NIST 98, as well as home-made MS databases.

Structural organization of the photosynthetic apparatus – the thylakoid membranes were isolated according to (Dobrikova et al., 2003). The concentration of the samples was 20 μg chl.ml^{-1} as determined by the method of Arnon (1949). 77K fluorescence emission spectra were recorded on Jobin Yvon JY3 spectrofluorimeter upon 436 nm and 472 excitation using 4 nm excitation and emission slits.

Cytokinin analysis – the freeze-dried samples (equivalent to 9 to 27 mg dry weight) were extracted and purified according to Dobrev and Kamínek (2002). Internal deuterated standards were added to the samples prior to extraction. Using reverse phase and ion exchange chromatography two fractions were separated, (1) fraction A containing hormones of acidic and neutral character (auxins, abscisic acid, salicylic acid, jasmonic acid and gibberellins), and (2) fraction B containing the hormones of basic character (i.e. CKs). The CKs were quantified using HPLC (Ultimate 3000, Dionex) coupled to hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems) set in selected reaction monitoring mode.

Results and Discussion

Effect of plant growth regulators on terpenoid profiles of aerial and underground parts of *Artemisia alba in vitro* – The aerial parts of *A. alba* in the control media (GAIP_0) and in the media containing IBA (0.5 mg.L^{-1} and 1.0 mg.L^{-1} GAIP_1 and GAIP_2, respectively) were characterized by strong prevalence of total monoterpenoids (composed of oxygenated monoterpenes and irregular oxygenated monoterpenes) in comparison with sesquiterpenoids (Table 1). As previously reported these three media were typical by an extensive root system development (Danova et al., 2012). It was also demonstrated that 6 months after tissue culture initiation, the irregular oxygenated monoterpenes were mainly present in aerial parts of *A. alba* in GAIP_0 and GAIP_2 media (Danova et al., 2012). In this paper, it was established that prolonged tissue culture of *A. alba* shoots (18 months) led to the presence of irregular oxygenated monoterpenes in all media samples. However, oils from GAIP_0, GAIP_1 and GAIP_2 cultured plants were still characterized by domination of monoterpenoids (MO + IMO) over sesquiterpenoids (S + SO), while the oils of GAIP_3 and GAIP_4 plants were characterized by

Table 1

Terpenoid profile in the aerial parts of *Artemisia alba* plants grown *in vitro*. Terpenoids are presented as a total content of the components of the respective class: M – monoterpenoids (i.e. MO – oxygenated monoterpenes; IMO – irregular oxygenated monoterpenes); S – sesquiterpene hydrocarbons; SO – oxygenated sesquiterpenes

Culture medium	MO [%]	IMO [%]	S [%]	SO [%]	M total [%]	S total [%]	M/S ratio
GAIP_0	10	72.4	10	5.8	82.4	15.8	5.2
GAIP_1	11.3	69.3	10	5.5	80.6	15.5	5.2
GAIP_2	12	71	10.6	5.4	83	16	5.2
GAIP_3	6	64.3	20.5	7.4	70.3	27.9	2.5
GAIP_4	6.2	59.1	24.6	7.6	65.3	33.1	2

Table 2
Terpenoid profile in the underground parts of *Artemisia alba* plants grown *in vitro*. IMO – irregular oxygenated monoterpenes; S – sesquiterpene hydrocarbons; SO – oxygenated sesquiterpenes

Culture medium	IMO [%]	S [%]	SO [%]	S total [%]	M/S ratio
GAIP_0	0.9	26.8	55	81.8	0.01
GAIP_1	1.5	25.2	58.3	83.5	0.02
GAIP_2	1.1	23.3	54.9	78.2	0.01
GAIP_3	6.4	24.7	43.8	68.5	0.09
GAIP_4	10	18.4	40.8	59.2	0.17

sesquiterpenoid prevalence (Table 1; similarly to the plants in the same media 6 months after tissue culture initiation).

Unlike the aerials, very low levels of monoterpenoids (comprising only of irregular oxygenated monoterpenes) as compared with sesquiterpenoids were detected in underground parts of *A. alba* in all media (Table 2).

Thus, a very low ratio between mono- and sesquiterpenoids (0.01–0.17) was observed. In addition, in contrast to the aerial parts, monoterpenoid levels were considerably higher in the presence of BA in the media (GAIP_3 and GAIP_4). In GAIP_3 and GAIP_4 BA amendment led to extensive callusogenesis and suppression of root formation. Within three-month period followed in this work, only very rare rooting was detected in these two media types (data not shown).

The strong prevalence of sesquiterpenoids in the underground parts (especially their higher levels in the media with extensive root development – GAIP_0, GAIP_1 and GAIP_2) together with their dominance in the aerial parts of GAIP_3 and GAIP_4 raises the question of either a possible translocation of these components from the aerials towards the roots or of an existence of separate biogenetic pathways in these different organs.

Effect of PGR on the structural organization of photosystem II – It is well established that photosystem II is highly sensitive to environmental stress factors and inherent growth and development regulators, being enrolled in several protective mechanisms. The peripheral antennae of photosystem II as a major light harvesting complex of photosystem II (LHCII) represent a main actor in a variety of stress responses; its oligomerization state is known to change upon light, heat and osmotic stress. 77K fluorescence spectroscopy was used to follow the oligomerization of LHCII upon PGR supplementation since the ratio between the fluorescence intensities at 693 nm and 685 nm (F693/685 nm) correlates with the formation of LHCII aggregates. The comparison between the typical 77K fluorescence spectra of thylakoid membranes isolated from GAIP_0 and GAIP_4 cultured plants (characterized by the domination of monoterpenoids and sesquiterpenoids in the aerials, respectively) shows a significantly lower ratio F693/685 nm for GAIP_4 compared to the control GAIP_0 (Figure 1A). Analysis of the F693/685 nm for the different media indicates that the extensive root development, accompanied

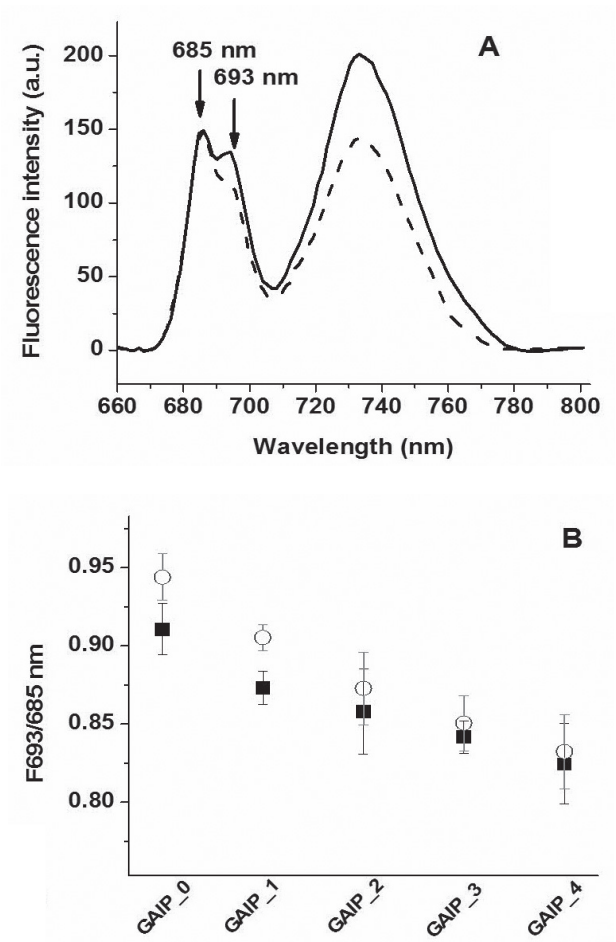


Fig. 1. (A) Typical 77K fluorescence spectra of thylakoids isolated from GAIP_0 (solid line) and GAIP_4 (dashed line) recorded upon 436 nm excitation. For clarity the 685 nm and 693 nm emission bands are indicated by arrows. (B) Averaged F693/685 nm ratio (and its standard error) determined for the studied variants upon chlorophyll *a* (436 nm, ■) and *b* (472 nm, ○) excitation

Table 3
Levels of endogenous *trans*-zeatin, dihydrozeatin and their ribosides in *A. alba* plants grown *in vitro*

Sample	*Trans*-zeatin [pmol/gFW]	*Trans*-zeatin riboside [pmol/gFW]	Dihydrozeatin [pmol/gFW]	Dihydrozeatin riboside [pmol/gFW]
GAIP_0 aerials	11.47^{b}	26.43^{d}	123.97^{c}	0.96^{a}
GAIP_0 roots	17.95	18.57^{c}	5.46^{a}	3.24^{c}
GAIP_1 aerials	6.19^{a}	27.5	114.14^{c}	0.98^{a}
GAIP_1 roots	91.38	6.56	4.34^{a}	0.91^{a}
GAIP_2 aerials	9.93^{b}	42.9	274.13	3.01^{c}
GAIP_2 roots	78.63	9.88^{b}	5.25^{a}	0.77^{a}
GAIP_3 aerials	6.85^{a}	12.53^{b}	12.98	0.64^{a}
GAIP_3 roots	11.63^{b}	22.32^{d}	15.79	1.93^{b}
GAIP_3 callus	10.64^{b}	2.09^{a}	15.21^{b}	2^{b}
GAIP_4 aerials	10.19^{b}	16.36^{c}	59.71	0.89^{a}
GAIP_4 roots	11.56^{b}	29.39	5.55^{a}	0.95^{a}
GAIP_4 callus	8.41^{ab}	4.4^{a}	15.98^{b}	3.59^{c}

Same letters denote non-significant differences ($p < 0.01$)

Table 4
Levels of endogenous *cis*-zeatin, N^6-(2-isopentenyl)adenine and their ribosides in *A. alba* plants grown *in vitro*

Sample	*cis*-zeatin [pmol/gFW]	*cis*-zeatin riboside [pmol/gFW]	N^6-(2-isopentenyl) adenine [pmol/gFW]	N^6-(2-isopentenyl) adenosine [pmol/gFW]
GAIP_0 aerials	9.47^{a}	42.76^{c}	6.5^{b}	32.38
GAIP_0 roots	9.65^{a}	15.33^{b}	1.93	8.41
GAIP_1 aerials	8.06^{a}	25.0	18.37	24.45
GAIP_1 roots	8.53^{a}	18.05	3.75 ^{a}	4.03^{a}
GAIP_2 aerials	14.61^{a}	76.15	10.18^{c}	67.01
GAIP_2 roots	5.41	14.28^{b}	3.36^{a}	5.46^{b}
GAIP_3 aerials	8.09^{a}	58.9^{c}	7.06^{b}	47.01
GAIP_3 roots	9.88^{a}	13.93^{a}	10.15^{c}	6.34^{b}
GAIP_3 callus	11.69^{b}	14.15^{ab}	7.83^{b}	4.35^{a}
GAIP_4 aerials	10.52^{b}	50.77^{c}	9.08^{b}	38.55
GAIP_4 roots	12.75^{b}	16.16^{ab}	2.62^{a}	5.28^{a}
GAIP_4 callus	13.57^{b}	11.46^{a}	5.32^{ab}	3.74^{a}

Same letters denote non-significant differences ($p < 0.01$)

by strong domination of the monoterpenoids in the aerials (GAIP_0, GAIP_1 and GAIP_2) was associated with a higher degree of aggregation of LHCII (higher F693/685 nm, Figure 1B). On the contrary, sesquiterpenoid domination, intensive callusogenesis, rare rooting and extensive aerial parts development (GAIP_3 and GAIP_4) was associated with a decrease in LHCII aggregation (lower F693/685 nm, Figure 1B). Thus, for the first time we demonstrate a relation between the macromolecular organization of photosystem II and the factors affecting the terpenoid biosynthesis in *A. alba*.

Those attributes are shown to be associated with the morphological development of plants as a response to the different PGR additions.

Effect of plant growth regulators on endogenous cytokinin levels – Different PGR treatments considerably affected contents of endogenous CKs in *A. alba* plants *in vitro* (Tables 3 and 4). Well-developed root system in control GAIP_0 medium as well as stimulated rooting in the presence of IBA (GAIP_1 and GAIP_2 media) were associated with increased levels of CKs *trans*ZR, DHZ and DHZR as well as of iP; (with the exception of control GAIP_0 medium where comparable iP levels with GAIP_3 were observed) in the aerial parts and to striking raise of *trans*Z concentration in the roots of *A. alba*. Addition of exogenous BA (0.2 $mg.L^{-1}$) resulted in decreased contents of these bioactive endogenous CKs in the aerials (GAIP_3 and GAIP_4 media).

It is well known that the first committed step in CK biosynthesis is an addition of the isopentenyl side chain from DMAPP, which serves also as a precursor of isoprene in terpenoid biosynthesis, to the adenosine moiety.

Thus, the observed relations between the reduction of sesquiterpenoid levels (which are biosynthesized at a more developed stage after monoterpenoids in the biogenetic pathway) and some of the studied endogenous CKs impose the question of a possible overlapping of terpenoid biosynthetic pathways and isoprenylation of CKs as a factor affecting terpenoid biogenesis of the essential oil of the species.

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