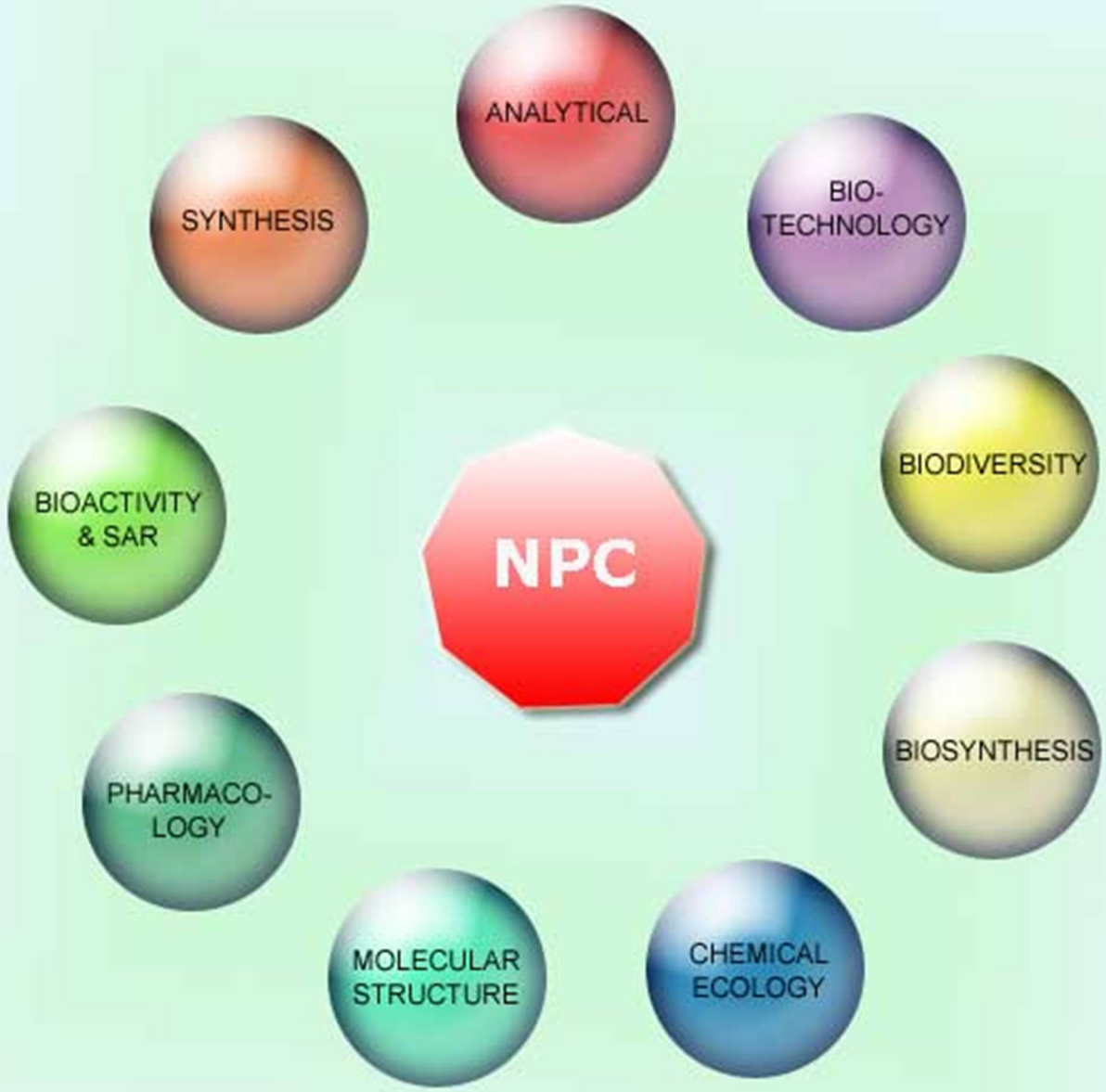
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Cytokinin and Auxin Effect on the Terpenoid Profile of the Essential Oil and Morphological Characteristics of Shoot Cultures of *Artemisia alba*

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The influence of plant growth regulators (PGR) on the essential oil composition and *in vitro* development of *A. alba* shoot cultures was studied. Two types of oils were determined, based on their terpenoid content. Close relations between the morphogenetic effects of PGR and the essential oil profile of the species were observed. Predominance of root over shoot development was connected with a drop in the amounts of sesquiterpenoids and the direction of biosynthesis towards oxygenated monoterpenoids in the PGR-free control medium, as well in those with 1.0 mg L^{-1} indole-3-butyric acid (IBA) supplementation. On the contrary, lack of root formation and stimulated callusogenesis caused by the addition of 0.2 mg L^{-1} 6-benzyladenine (BA), irrespectively of the presence of IBA (either 0.5 mg L^{-1} or 1.0 mg L^{-1}), resulted in the strong prevalence of sesquiterpenoids in the oils. These results are indicative that the morphological development of *A. alba* shoot cultures affects the terpenoid biosynthetic pathway bringing out the hypothesis for a possible root to shoot signaling.

Keywords: *Artemisia alba,* Shoot cultures, Essential oil profile, Irregular monoterpenoids, Plant growth regulators, Morphological characteristics.

The aerial parts of *Artemisia alba* Turra have been traditionally utilized as a stomach digestive and tonic in the form of a decoction [1a]. Essential oil of the species has been shown to possess strong spasmolytic and antimicrobial activities [1b,c]. The literature survey revealed a great variability in the essential oil composition [2a-c]. *In vitro* culture is a controlled model system, suitable to study the biosynthesis of target secondary metabolites independently of environmental factors. However, to the best of the authors' knowledge, there is only scarce information on tissue culture of *A. alba* so far. Available data concern comparison of the essential oils obtained from *ex vitro* and *in situ* samples of *A. alba* [1b]. In this work we report the occurrence of two types of essential oils differing in the monoterpenoids/sesquiterpenoids ratios, determined by the morphogenetic effect of plant growth regulators (PGR) in shoot cultures of *A. alba.* Five media variants were compared: GAIP_0 (PGR-lacking control), GAIP_1 (0.5 mg L^{-1} IBA-supplemented), GAIP_2 (1.0 mg L^{-1} IBA), GAIP_3 (0.2 mg L^{-1} BA + 0.5 mg L^{-1} IBA) and GAIP_4 (0.2 mg L^{1} BA + 1.0 mg L^{-1} IBA).

Effect* of *IBA and BA on essential oil composition of* A. alba *shoots: Five essential oils obtained from *A. alba* shoots were analyzed by GC and GC/MS. Comparison of the oil composition was based on up to 34 components (Table 1) in concentrations exceeding 0.5% in at least one of the samples, comprising: 85.9% in GAIP_0; 85.1% in GAIP_1; 84.4% in GAIP_2; 84.7% in GAIP_3 and 79.2% in GAIP_4 media. Oxygenated monoterpenoids (MO) dominated over sesquiterpenoids (S) in the control (GAIP_0) and in media lacking cytokinin supplementation (GAIP_1 and GAIP_2). Significant levels of irregular monoterpenoids (IMO) were found in the control and in plants with high (1.0 mg L^{-1}) IBA supplementation (GAIP_2) (Table 1). The addition of 0.2 mg L^{-1} BA to the culture medium (GAIP_3 and GAIP_4) resulted in a drop of MO, depletion of IMO and prevalence of sesquiterpenoids in the oils (Table 1). Thus, the results from the conducted experiments showed the occurrence of two essential oil types based on the presence of IMO and variation of the MO/(S + SO) ratio. The first oil type (isolated from GAIP_3 and GAIP_4) was characterized by a higher concentration of sesquiterpenoids (S+SO), of which germacrene D and bicyclogermacrene were dominant, while IMO were either only negligible (GAIP-4) or lacking (GAIP-3). The second oil type (GAIP_0 and GAIP_2) was characterized by an increase in oxygenated monoterpenes (MO), of which IMO were in a significant percentage (Table 1), and a low amount of germacrene D and bicyclogermacrene. It was interesting that similar to GAIP_0 and GAIP_2, MO were also dominant in the essential oil of GAIP_1, but IMO were absent in the latter medium variant. Furthermore, S, especially the amounts of germacrene D and bicyclogermacrene, were significantly higher in GAIP_1, as in GAIP_3 and GAIP_4. So, the GAIP_1 essential oil exhibited features characteristic for both oil types described above.

Morphogenetic effect of PGR: Supplementation with 0.5 mg L^{-1} IBA led to only slight increase in primary shoot length in comparison with the control (Table 2). Increase of IBA concentration to 1.0 mg L^{-1} resulted in further rise in shoot number and compactness (IC). Addition of 0.2 mg L^{-1} BA led to the formation of a higher number of axillary shoots with compressed length (Table 2). The used PGR also stimulated secondary axillary shoots formation, which were absent in the control (Table 2). Their length and number were higher in the BA supplemented media, while their compactness was highest in the 1.0 mg L^{-1} IBA supplemented GAIP_2 (Table 2). Supplementation with IBA stimulated root formation in GAIP_1 and GAIP_2, as induced roots were characterized by higher number and thickness, but lower length in comparison with the control (Table 3). Increased IBA concentration also stimulated the formation of root branches, thus markedly enhancing the overall area of the root system. Furthermore, 1.0 mg L^{-1} IBA also stimulated aerial parts development, thus raising the shoot/root length when compared with GAIP_0 and GAIP_1 (Table 3).

Table 1: Essential oil composition of *A. alba* shoot cultures in the five media.

RI	Compounds	Type	GAIP_0	GAIP_1	GAIP_2	GAIP_3	GAIP_4
984	β – Pinene	M	-	0.8	0.5	0.5	0.4
1000	Yomogi alcohol	IMO	28.8	-	9.1	-	0.7
1037	1,8-Cineole	MO	0.3	12.3	8.5	7.1	8.4
1064	γ-Terpinene	M	-	0.4	0.3	0.4	0.4
1071	*cis*-Sabinene hydrate	MO	0.4	2.3	2.3	1.3	2.1
1085	Artemisia alcohol	IMO	11.5	-	4.7	-	-
1106	*trans*-Sabinene hydrate	MO	0.6	1.8	1.6	1.1	1.4
1129	*cis*-*p*-Menth-2-en-1-ol	MO	0.2	0.7	0.4	0.2	0.5
1133	Unidentified	MO	0.6	-	0.5	-	-
1156	Camphor	MO	1.8	18.3	12.5	14.1	15.4
1169	*cis*-Chrysanthenol	MO	0.9	1.2	0.4	0.5	0.7
1171	Pinocarvone	MO	1.1	1.8	0.7	1.1	1.2
1173	Artemisyl acetate	IMO	8.4	-	7.1	-	-
1174	Borneol	MO	0.7	1.8	1.2	1.5	1.7
1180	*cis*-Linalooloxide	MO	-	0.6	0.5	+	0.4
1185	Terpinen-4-ol	MO	-	0.9	0.6	0.5	0.6
1197	α-Terpineol	MO	0.9	-	0.4	-	-
1287	Unidentified	MO	1.1	-	0.7	-	-
1326	Unidentified	MO	0.9	-	0.8	-	-
1349	δ-Elemene	S	1.6	5.1	3.4	3.9	4.5
1403	β-Elemene	S	0.5	3.6	1.6	7.9	4.1
1433	β-Caryophyllene	S	0.4	0.7	0.6	1.1	0.9
1460	*trans*-β-Farnesene	S	2.8	+	1.1	2.6	1.2
1492	Germacrene D	S	11.4	24	15.9	30.2	26.2
1505	Bicyclogermacrene	S	0.9	2.2	1.5	2.6	2.4
1532	δ-Cadinene	S	0.4	0.3	0.3	0.5	0.5
1560	Elemol	SO	3.2	2.7	3.3	3	2.4
1575	E-Nerolidol	SO	-	0.4	0.2	1.8	0.7
1621	Caryophyllene oxide	SO	2.1	0.5	0.6	-	+
1636	*epi*-α-Cadinol	SO	0.6	0.3	0.5	+	+
1688	Elemol acetate	SO	1.6	-	0.6	-	-
1696	α-Bisabolol	SO	-	2.4	1.6	2.8	2.4
1775	Unidentified	SO	2.2	+	0.4	+	+
	Total		85.9	85.1	84.4	84.7	79.2

M – monoterpene hydrocarbons; MO – oxygenated monoterpenes; IMO irregular oxygenated monoterpenes; S – sesquiterpene hydrocarbons; SO – oxygenated sesquiterpenes.

Table 2: Morphoregulatory effect of PGR supplementation to formation of aerial parts of *A. alba*.

Medium	Primary axillary shoots			Secondary axillary shoots		
	Number	Length [cm]	IC	Number	Length [cm]	IC
GAIP_0	3.9 ±0.3	3.4 ±0.2	4.1 ±0.3	-	-	-
GAIP_1	4.1±0.2	4.4 ±0.3	3.8 ±0.2	3.4 ±0.5	0.6 ±0.1	6.5 ±0.5
GAIP_2	5.1 ±0.5	3.9 ±0.5	4.6 ±0.3	3.3 ±0.4	0.6 ±0.1	8.4 ±0.7
GAIP_3	10.6 ±0.9	2.8 ±0.2	4.8 ±0.2	8.8 ±0.8	1.04 ±0.2	7.4 ±0.5
GAIP_4	6.7 ±0.1	3.2 ±0.1	4.5 ±0.1	8.8 ±0.4	0.98 ±0.1	7.5 ±0.3

The values are represented as means ±SE.

Root formation was suppressed in 0.2 mg L^{-1} BA containing media in spite of the exogenously supplied auxin, even at its high (1.0 mg L^{-1}) concentration in GAIP_4. The results demonstrate a close relationship between the terpenoid profile of the oils and the morphological development modified by PGR in *A. alba* shoots. Extensive root development was shown to be connected to the prevalence of MO with significant quantities of IMO in the oils. On the other hand, inhibited rooting (with the formation of only callus in the shoot base) and stimulated aerial parts development led to depletion of IMO and raising of sesquiterpenoids levels in the samples. The obtained two types of essential oil in the present work correlate with the reported data [2c] for *A. alba in situ.* Noteworthy,

Table 3: Morphoregulatory effect of PGR supplementation to root formation and root/shoot development in *A. alba*.

Medium	Primary roots		Root branches		Root/shoot ratios	
	Number	Length [cm]	Number	Length [cm]	Number	Length [cm]
GAIP_0	5.9 ±0.3	8.3 ±0.5	6.2 ±2.1	1.3 ±0.6	0.7 ±0.1	0.5 ±0.0
GAIP_1	9.6 ±0.8	5.1 ±0.3	4.7 ±2.0	1.4 ±0.5	0.7 ±0.1	0.9 ±0.1
GAIP_2	17.7 ±1.9	2.9 ±0.3	7.5 ±2.0	0.5 ±0.1	0.6 ±0.1	1.6 ±0.2

Root formation absent in media GAIP_3 and GAIP_4. The values are represented as means ±SE.

in the present work, the two oil types were obtained under principally different conditions of modification of PGR supplementation *in vitro*. It could be expected that the decisive factors for terpenoid biosynthesis might be identical in these cases.

Experimental

In vitro* culture of *A. alba: Shoot cultures were initiated from 0.1% $HgCl_2$ surface sterilized stem segments of the aerial parts of a field grown commercial cultivar of *A. alba* (SOM – 167 590) in culture medium containing the basic Murashige and Skoog (MS) [3] salts, Gamborg's [4a] vitamin supplementation, 15 g L^{-1} sucrose, 6.5 g L^{-1} agar and 0.5 mg L^{-1} benzyl adenine (BA). Multiple axillary shoots were induced and further maintained on MS salts and G5 vitamins PGR-free medium, supplemented with 30 g L^{-1} sucrose, at 25ºC and a 16/8 h photoperiod and a 45 days period of regular subculture. Five media variants were compared: the control medium (GAIP_0); 0.5 mg L^{-1} IBA supplemented control (GAIP_1); 1.0 mg L^{-1} IBA supplemented control (GAIP_2); supplementation of 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). At least 15 stem explants (with 3 nodes per explant) of *in vitro* cultured *A. alba* shoots were grown in each medium variant for 8 weeks under the conditions described above. Length [cm] and number of primary and secondary axillary shoots, as well as of roots and root branches were estimated for at least 15 separately growing plants. Index of compactness (IC) was calculated as the number of nodes per cm of shoot length.

Essential oil preparation and GC and GC/MS analyses: The essential oil was prepared by micro steam distillation-extraction of the fresh shoots of the *in vitro* grown plants in a modified Lickens-Nickerson apparatus for 2.5 h [4b] using diethyl ether as a solvent. GC analysis was performed under the experimental conditions reported earlier [5a]. The individual components were identified by their RI, referring to known compounds from the literature [5b,c], and also by co-comparison of their MS, with those of NIST 98, as well as home-made MS databases.

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