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In vitro culture development and polyphenolics production of *Artemisia alba* Turra

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ABSTRACT

Artemisia alba Turra is an aromatic plant, characterized by a high variability of the terpenoid profile of its essential oil. In previous research, *in vitro* shoots of the plant were developed, aiming at elucidation of the effects of plant growth regulators on essential oil production. Though less information is available in literature regarding the non-volatile components of the plant, a number of works report on the presence of compounds with coumarin, flavonoid and sesquiterpene structure which might attribute to the pharmacological activity of the plant.

In the present work, different lines of differentiated and non-differentiated *in vitro* cultures of the plant have been developed in solid and liquid media. The potential of these lines to produce compounds with phenolic and flavonoid structure has been studied. In differentiated shoot cultures, low benzyl adenine (BA) concentration alone or in combination with different indole-3-butyric acid (IBA) concentrations increased the polyphenolic levels as compared with plant growth regulators free control, as well with media with high BA alone or combined with IBA. The content of these compounds was also low when IBA was applied alone. In non-differentiated cell aggregate cultures, 1-naphthaleneacetic acid (NAA) in combination with BA significantly increased polyphenolics as compared with IBA. Observations on the morphology of the aggregates formed in the two media suggested that the more compact structure and larger size of aggregates as a result of NAA supplementation might be decisive for the higher polyphenolics productivity, as compared with IBA.

Keywords: *Artemisia alba* Turra in vitro shoot culture, morphogenesis in vitro, cell aggregate cultures, polyphenolics production.

Introduction

Artemisia alba Turra is a fragrant shrub, distributed in Southern Europe, traditionally applied as tonic and digestive in the form of decoction. Research work has shown the anti-inflammatory and spasmolytic activity of extracts of the plant as well as the antimicrobial activity of its essential oil (Radulović and Blagojević, 2010 and references cited within). Less information is available on the non-volatile constituents of the plant as nerolidol derivatives, coumarins and flavonoids have been isolated and identified in its aerials (Maggio et al., 2011 and 2013). A recent research has revealed the presence of ten new sesquiterpene alcohols in the plant (Todorova et al., 2014). *In vitro* cultures of the plant have been previously established with the purpose of investigation of essential oils production in controlled laboratory conditions. The terpenoid profile of the plant *in vitro* has been studied, leading to the development of two distinctive systems for the yield of essential oils with either monoterpenoid or sesquiterpenoid domination (Danova et al., 2012). The aim of the present work was to develop different

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in vitro culture systems for the controlled production of secondary metabolites with polyphenolic structure.

Materials and Methods

Plant material

Shoot cultures were initiated through surface sterilization of stem explants of field grown *A. alba* (Danova et al., 2012). After establishment of the cultures on benzyl adenine BA supplemented medium, stocks were kept on plant growth regulators (PGR) free medium supplemented with the Murashige and Skoog (Murashige and Skoog, 1962) macro- and micro-salts medium, modified with Gamborg (Gamborg et al., 1968) vitamins and 2 g/l glycine supplementation. Sucrose was added at 30 g/l concentration, and media solidified with 6.5 g/l agar. Plants were growth at 25 ± 0.2 °C at 16/8 h photoperiod.

Plant growth regulators treatments

For the study of the effect of PGR on developmental patterns of shoot cultures, *in vitro* excised stem explants comprising of 3-4 nodes were cultivated in media with the following PGR modifications: GAIP_0 - control PGR-free medium; GAIP_1 - 0.5 mg/l indole-3- butyric acid (IBA); GAIP_2 - 1.0 mg/l IBA; GAIP_3 - 0.2 mg/l benzyl adenine (BA) + 0.5 mg/l IBA; GAIP_4 - 0.2 mg/l BA + 1.0 mg/l IBA; GAIP_5 - 0.2 mg/l BA; GAIP_6 - 0.7 mg/l BA; GAIP_7 - 0.2 mg/l BA + 0.8 mg/l IBA; GAIP_8 - 0.7 mg/l BA + 0.5 mg/l IBA; GAIP_9 - 0.7 mg/l BA + 1.0 mg/l IBA supplemented media. Media were solidified with 6.5 g/l agar and *A. alba* shoots were grown for 10 weeks at 25±0.2°C and a 16/8 h photoperiod.

For the study of the morphogenic response of different explant types to PGR, leaf (1), stem (2) and root (3) explants were inoculated (abaxial facing the medium) into the following media: (I) 1 mg/l 2,4-D; (II) 1 mg/l NAA; (III) 1 mg/l BA; (IV) 1 mg/l BA + 0.1 mg/l NAA and (V) 1 mg/l BA + 0.1 mg/l 2,4-D. Media were solidified with 6.5 g/l agar and explants were kept in the dark at 25±0.2°C. Morphogenesis was recorded 8 weeks after explant inoculation.

For establishment of callus culture root explants were placed into MS medium, supplemented with 0.1 mg/l BA + 1.5 mg/l IBA, supplemented with 20 g/l sucrose and solidified with 6.5 g/l agar (ER_3). Explants were kept in the dark and after callus induction, cell aggregates were transferred and further on maintained into liquid media with the following PGR supplementations: ER_3 (formulation as described above), ER_3_NAA (0.1 mg/l BA + 1.5 mg/l NAA). These two modifications were cultivated in the dark on rotary shaker, 100 rpm, at 25±0.2°C.

Microscopic imaging of the obtained structures

Microscopic imaging of regenerated structures and cell aggregates, grown in the liquid culture was done by Leica M60 Microscope; photographs were taken by means of Leica IC80 HD Camera and images processed by Leica Application Suite software.

Determination of malondialdehide and hydrogen peroxide in vitro

120 mg FW of the shoots were homogenized in a mortar at 4 °C with 0.1% trichloroacetic acid and centrifuged for 20 min at 15 000 rpm. For malondialdehyde (MDA) estimation, an aliquot of the supernatant was mixed with phosphate buffer pH 7.4 and after the addition of 0.5 % thiobarbituric acid (in 20 % trichloroacetic acid), the samples were boiled for 30 min (Dhindsa et al., 1981). After rapid cooling of the samples in an ice-bath, absorption was measured at λ = 532 and 600 nm using the extinction coeficient 155 mM^{-1} cm^{-1} (Heath and Packer, 1968). For the hydrogen peroxide (H_2O_2) assay, an aliquot of the supernatant was mixed with phosphate buffer pH 7.4 and after the addition of 1 M KI, samples were incubated in the dark for 60 min and absorption was measured at λ = 390 nm. The content was calculated using a standard curve of H_2O_2 in the range of 1–100 nmol/ml of hydrogen peroxide (Jessup et al., 1994).

Determination of total phenolic and flavonoid content

100 mg DW of the plant material were extracted with 80% (v/v) hot ethanol and then centrifuged at 15 000 rpm for 15 min. Total phenolics were determined by the Folin & Ciocalteu's colorimetric method of Singleton et al. (1999). The absorbtion was measured at λ = 730 nm and the total phenolics were calculated by means of a calibration curve of chlorogenic acid and expressed as mg of chlorogenic acid equivalent per 1 g DW of the sample. Total flavonoids content was determined using a colorimetric assay in accordance with the method of Zhishen (1999). The absorption at λ = 510 nm was measured and the concentration was calculated by means of a calibration curve of (+)catechin. The total flavonoids of the samples were expressed in mg of (+)catechin equivalent per 1 g DW of the

sample. All measurements were performed in triplicate with three repetitions.

Results

Effect of PGR on shoot cultures development

Shoots grown in PGR-free control, as well as in media supplemented with IBA (GAIP_0, GAIP_1 and GAIP_2) exhibited the development of both shoot and root system (Figure 1 A).

Figure 1. *Shoot and root development in PGR-free control (GAIP_0) (A); Stimulation of root number and inhibition of root length by 0.5 mg/l IBA supplementation in GAIP_1 (B); Stimulation of axillary shoot formation, inhibition of rooting and stimulation of callusogenesis at the shoot-clumps base by supplementation of 0.2 mg/l BA in combination with the 0.5 mg/l IBA treatment GAIP_3 (C); Further stimulation of axillary shoot formation and scarce indirect root formation (r) in 0.7 mg/l BA and 1.0 mg/l IBA supplementation in GAIP_9 (D). Space bar = 1cm.*

Roots in 0.5 mg/l IBA supplemented plants (GAIP_1) were high in number but shorter in length as compared with GAIP_0 and GAIP_2 (Figure 1B). Then the addition of 0.2 mg/l BA to these two IBA supplementations led to inhibition of rooting and profound callusogenesis at the base of the explants (GAIP_3 and GAIP_4, Figure 1 C). Further on, the single application of BA (GAIP_5 and GAIP_6), as well as combinations of higher BA and IBA concentrations also led to predominant callusogenesis at the base of explants, with only scarce indirect rooting through the initially formed callus tissue (Figure 1 D).

Effect of PGR on morphogenesis of A. alba explants

The morphogenic response of the three explant types to PGR is illustrated in Figure 2. Leaves responded with callusogenesis upon auxin treatment (2,4-dichlorophenoxyacetic acid and α-naphtylacetic acid), and with callusogenesis and indirect shoot formation to combination of the both auxins with benzyl adenine.

The individual application of benzyl adenine did not lead to any morphogenesis, but to necrosis of the explants. Stem segments responded with callusogenesis upon 2,4-dichlorophenoxyacetic acid and to its combination with benzyl adenine, callusogenesis and rooting upon α-naphtylacetic acid, auxillary shoots and callus formation upon α-naphtylacetic acid and benzyl adenine, as well as individual benzyl adenine treatments. Root segments responded with callusogenesis to 2,4-dichlorophenoxyacetic acid, its combination with benzyl adenine, and with rooting to α-naphtylacetic acid and its combination with benzyl adenine. Benzyl adenine alone did not induce marked morphogenic response of the root explants.

Effect of explant type on the malondialdehyde and hydrogen peroxide levels in vitro

Comparison between the levels of malondialdehyde (as a level of lipid peroxidation) in the aerial and root samples of PGR-free plants showed considerably lower levels of this parameter in the roots as compared with the aerials. Levels of hydrogen peroxide were slightly higher in the roots as compared with the aerials of the plants (Table 1).

Effect of PGR on morphogenesis of A. alba cell aggregates in liquid media

Cell aggregates selected on 0.1 mg/l BA and 1.5 mg/l IBA medium (ER_3) had friable structure and were lighter in color as compared with aggregates grown in ER_3_NAA medium (Figure 3). The latter were compact, had smooth surface and with nearly iso-diametric proportions.

Effect of in vitro culture type on the polyphenolic content of A. alba

Polyphenolics production in A. alba in vitro shoots was affected by the application of PGR (Figure 4). Thus, highest levels of these compounds were observed in media with low levels of BA supplemented alone (GAIP_5) or in combination with medium (GAIP_3) and high (GAIP_4 and GAIP_7) IBA concentrations. The only case of elevated polyphenolics in high BA concentration was when it was combined with also a higher IBA concentration (GAIP_9).

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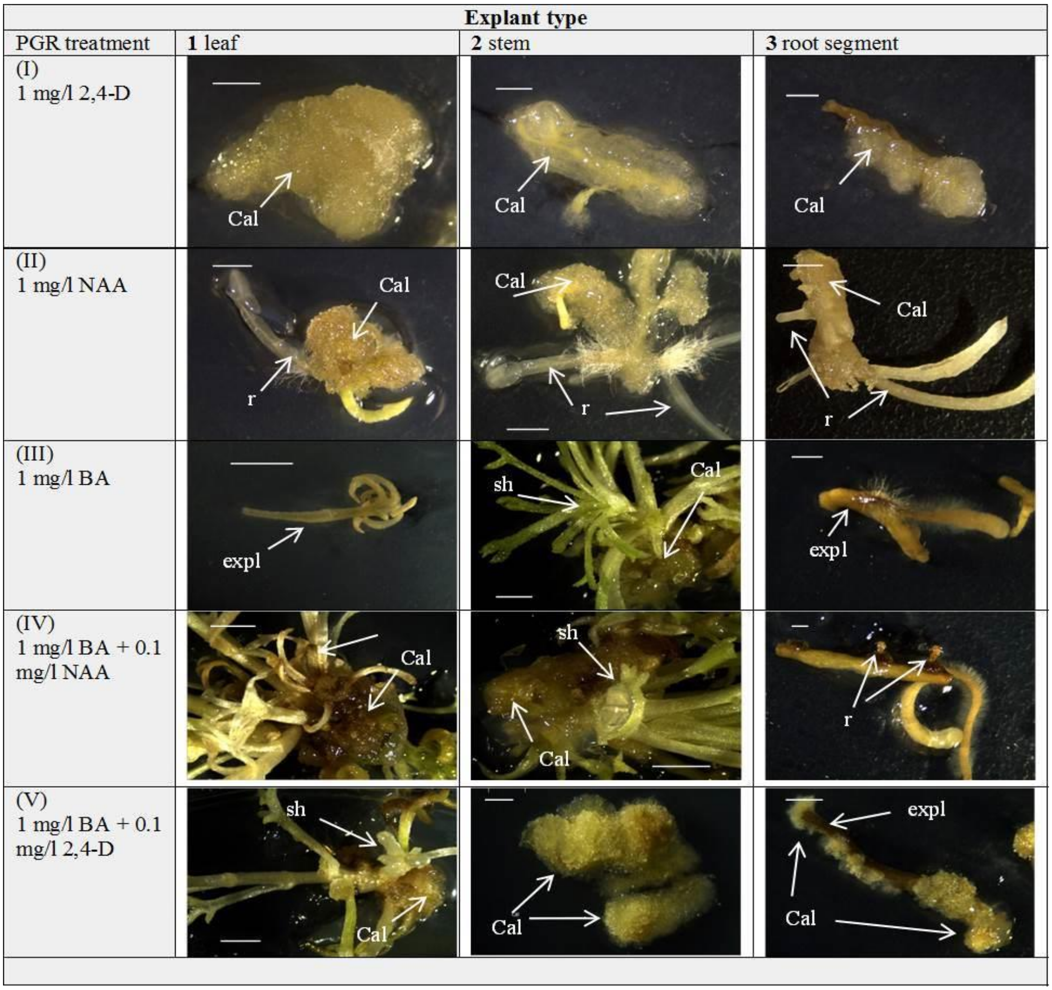

Figure 2. *Effect of PGR on the morphogenic potential of different explant types of Artemisia alba Turra. (Cal – callusogenesis, r – root formation, sh – shoot formation, expl – explant), space bar = 2mm.*

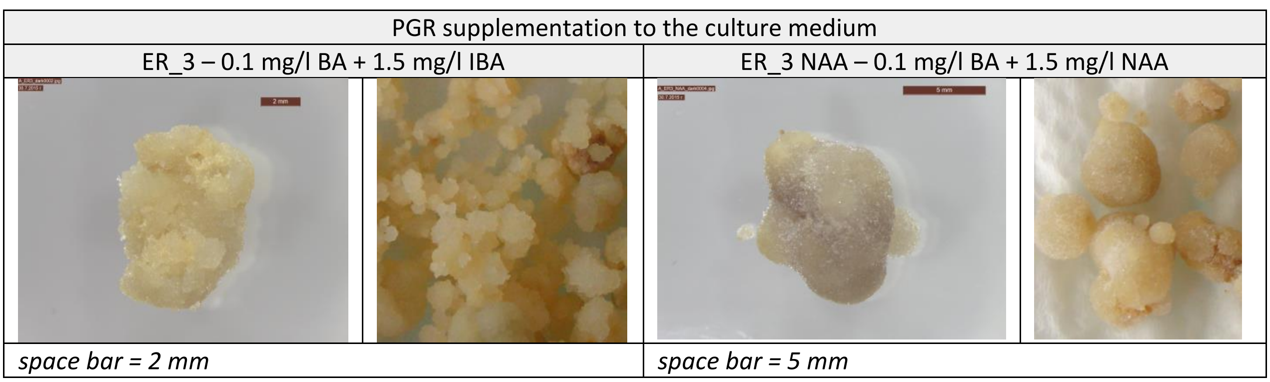

Figure 3. *Cell aggregate lines of Artemisia alba Turra selected through PGR supplementation.*

Table 1. *Malondialdehyde and hydrogen peroxide levels in aerial and root samples of Artemisia alba Turra in vitro.*

	MDA [nmol/g FW]	H_2O_2 [nmol/g FW]
Shoots	0.08 ± 0.001	0.61 ± 0.02
Roots	0.032 ± 0.01	0.85 ± 0.07

(±) values represent SE of three measurements.

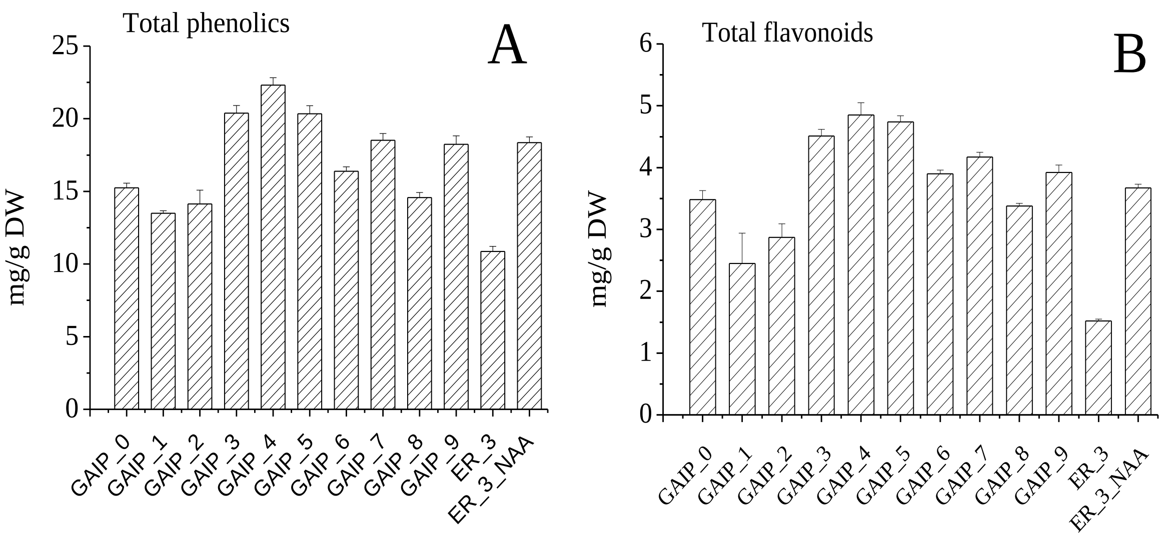

Figure 4. *Polyphenolic content of the different Artemisia alba Tura in vitro lines.(± values represent SE of six measurements)*

Interestingly, lower polyphenolics levels in shoots seemed to be related to either rooting (GAIP_0, GAIP_1 and GAIP_2) or high BA concentration applied alone (GAIP_6) or in combination with lower IBA treatment (GAIP_8). Levels of polyphenolics in ER_3_NAA cell aggregates, cultivated in liquid medium were significantly higher than the untreated differentiated control shoots (GAIP_0). Unlike them, IBA seemed to affect polyphenolic levels in adverse way, leading to a drop of these compounds in ER_3 cultivated cell aggregates.

Discussion

Cytokinins and auxins play a major role in controlling plant growth and development. They might act either synergistically or antagonistically in developmental processes in plant organism (Su et al., 2011). In the present study different cytokinin and auxin concentrations were used to affect developmental patterns of differentiated shoot cultures in solid media and liquid media. In differentiated shoots the application of even a low BA treatment inhibited rooting and stimulated intensive callusogenesis even at combinations with high IBA concentrations. In addition, sterile excised explants of the plant have shown distinctively variable response to the treatment with BA, as well as the two auxin types applied (NAA and 2,4-D). Root explants were shown to have the most conservative response always forming non-differentiated callus as response of the treatments. In, addition roots tissue was also characterized by significantly lower levels of malondialdehyde *in vitro*. It is known that malondialdehyde is formed by destruction of lipid membranes by reactive oxygen species and is considered as genotoxic due to its ability to bind to DNA molecule (Marnett, 1999a, b). Therefore root explants were chosen for the initiation of non-differentiated cultures in liquid medium. Treatments were shown to significantly affect polyphenolic production both in differentiated and non-differentiated cultures. Noteworthy in non-differentiated cell aggregates compactness of the obtained structures significantly contributed for elevation of the levels of polyphenolics *in*

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vitro. Development of globular structures in liquid medium *in vitro* has been shown to promote secondary metabolite production in *H. perforatum* (Vardapetyan et al., 2000). Cell suspensions have been considered as either homologous or heterologous ones, differences being in terms of morphology and uniformity of cell types. The first type consists of fine mostly homogenous populations of cells and the latter one consists of different cell types (clusters and aggregates). Namely these type of cell aggregates have been shown to produce some desired secondary metabolites, having a state of partial differentiation (Kirakosyan et al., 2011). The obtained lines will further be developed in order to investigate the phytochemical composition of the secondary metabolites produced.

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