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Influence of Nutrient Medium Composition on *in vitro* Growth, Polyphenolic Content and Antioxidant Activity of *Alchemilla mollis*

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Alchemilla mollis (Buser) Rothm. (Rosaceae) is a high-mountain medicinal plant growing in the Balkan Peninsula, with only one population in Bulgaria. *Alchemilla* plants (Lady's mantle) are commonly used in traditional medicine for treatment of many gynecological diseases. The commercial drugs "Herba Alchemillae" induce a rapid regeneration of skin epithelium and have styptic and anti-inflammatory actions. Because of the high content of phenolic compounds (tannins and flavonoids) and the ecological plasticity of the species, field cultivation or *in vitro* biomass production of *A. mollis* are possible alternatives to its collection from nature. Four MS based nutrient media differing in the concentration of the minerals and supplemented with α-naphthaleneacetic acid (NAA) and benzylaminopurine (BAP) were tested in order to examine their influence on the shoot multiplication effectiveness and the antioxidant activity of *A. mollis*, and also the possible relation between these parameters under the conditions of *in vitro* culture. The cultures grown for two months on these media differed significantly in their multiplication rates ($p<0.001$), as well as in their morphological features – height, leaf color and root development. Methanol extracts of *in vitro* cultivated and *ex vitro* adapted and acclimated on Vitosha Mt. (1500 m a. s. l.) plants were analyzed for tannin and flavonoid content and for free radical scavenging activity. The contents of flavonoids and tannins in the *in vitro* cultures of *A. mollis* cultivated on the four tested media differed significantly ($p<0.05$). The highest flavonoid content was found in the shoots cultivated on the control MS medium, as well as in the *ex vitro* adapted plants. The antioxidant activity of the *in vitro* cultures correlated positively with the concentrations of the PGRs in the respective media, and the *ex vitro* adapted plants had the highest antioxidant activity (IC_{50} 13.1±1.9 μg/mL) commensurable with that of the commercial antioxidant butylated hydroxytoluene (BHT) used as a positive control, with an IC_{50} of 12.65 μg/mL.

Keywords: Lady's mantle, Plant growth regulators, Flavonoids, Tannins.

Alchemilla plants (Lady's mantle) are commonly used in traditional medicine for treatment of many gynecological diseases [1]. The drugs induce a rapid regeneration of skin epithelium and have styptic and anti-inflammatory actions. The main biologically active substances in Herba et rhizoma Alchemillae are the phenolic compounds – tannins and flavonoids [2]. Phenolics, in particular flavonoids, may act as phytoalexins, antifeedants, attractants for pollinators, protective agents against UV light, and they also have an important role in the defense mechanisms of plants under different environmental stress conditions [3]. One of the most useful medicinal properties of many phenolic compounds is their ability to scavenge free radicals [4]. The antioxidant capacity of plant extracts has been extensively assessed by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, which is a quick, reliable, and reproducible method [5].

The commercial drug "Herba Alchemillae" is a complex of several *Alchemilla* species. Among previously analyzed species growing in Bulgaria, *A. mollis* has the highest content of phenolic compounds together with *A. flabellata* and the Bulgarian endemic plant *A. jumrukczalica* [6]. Recently, eight flavonoid glycosides were isolated from the most effective extract fraction of *A. mollis* in terms of free radical scavenging activity: *cis*- and *trans*-tiliroside, rhodiolgin, hyperoside, isoquercitrin, miquelianin, sinocrassoside D_2, and a new compound identified as gossypetin-3-*O*-β-D-galactopyranosyl-7-*O*-α-L-rhamnopyranoside [7].

A. mollis (Buser) Rothm. (Rosaceae) is a high-mountain plant species growing in the Balkan Peninsula [8]. Its unique population in Bulgaria is located in the Central Balkan National Park, Dzhendema Reserve, at 1162 m a.s.l. [9]. The species is critically endangered in Bulgaria according to the IUCN criteria and is included in the Red Book of Bulgaria, the Red List of Bulgarian vascular plants, the Biological Diversity Act, and the Medicinal Plants Act [10].

The first trials to cultivate *A. mollis* under controlled conditions proved the high ecological plasticity of the species. Plants developed well at different altitudes: in the experimental plots in the Bulgarian mountains Vitosha and Rhodopes, both located at 1500 m a. s. l., as well as in the Sofia plain at about 500 m a. s. l. [9]. Furthermore, the contents of flavonoids and tannins in plants collected from localities situated at different altitudes in Romania between 800 and 1800 m a. s. l. were similar [11]. These results suggested that *A. mollis* could be introduced as a crop and grown on some hilly desolate lands, the area of which has increased in Bulgaria during the last decades. However, the establishment of a commercial plantation of *A. mollis* as an alternative to plant collection from the wild will require a large quantity of plant seedlings.

The *in vitro* techniques for rapid plant micropropagation are known as a suitable tool for production of thousands of plants in a short time. Large-scale clonal propagation was first applied to ornamental plants, but during the last decades it has been extended to about 1000 plant species including vegetable and fruit crops, medicinal and aromatic plants, and trees [12]. *In vitro* micropropagation is a very useful tool for mass multiplication of rare and threatened plant species, especially for medicinal plants with conservation status [13]. Commercial cultivation of micropropagated *Narcissus* grown

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as a raw material for galanthamine production in the UK and in the US, revealed the effectiveness of the process, resulting in 1200 bulbs produced in 18 months from one parent bulb [14]. Although machines are used for many processes such as cutting of initial explants, planting of *ex vitro* adapted plantlets in soil on the field, the cost of *in vitro* multiplied plants could be further reduced using bioreactors [15]. Presently, most of commercially produced *in vitro* plants are multiplied through the organogenic pathway in agar-gelled cultures; however, some important crops, and ornamental and medicinal plants are successfully propagated in bioreactors [12a, 15a]. In the case of medicinal plants, *in vitro* production of biomass in bioreactors could be an alternative to the establishment of field plantations; therefore, it is important to evaluate the biosynthesis of the desired secondary metabolites in the *in vitro* obtained plantlets.

It is worth mentioning that plant species often have specific requirements concerning cultivation conditions and medium composition, especially plant growth regulators (PGRs), therefore the determination of the optimal parameters for *in vitro* growth and biosynthesis of secondary metabolites is a subject of many experiments. In general, cytokinins are added to the nutrient media for shoot multiplication because of their ability to inhibit the apical dominance, which results in development of numerous shoots from the meristem tissues. However, an excessive concentration of cytokinins could affect the normal plant morphology and physiology causing difficulties in the stage of *ex vitro* adaptation of the plants [16]. Furthermore, the optimal mineral concentration of the medium could be different depending on the plant species and the cultivation conditions. The most commonly used MS medium [17] is a very rich and saline one; therefore, it is sometimes used with twice reduced macro elements. Thus, differentiation and proliferation of micropropagated ferns, gladiolus, *Nerine* nodular clusters and *Lilium* bulblets in bioreactors was better on half-strength MS medium [15a, 18]. Successful micropropagation of *A. mollis* was reported from axillary buds on several media containing different combinations of auxins and cytokinins [19], but no information was found on the *in vitro* biosynthesis of this plant's phenolic compounds.

Our recent studies showed a good propagation efficiency of *A. mollis in vitro* culture during one year of cultivation, enhanced by consecutive subcultivations on agar-solidified, MS-based medium supplemented with the most commonly used plant growth regulators: α-naphthaleneacetic acid (NAA) and benzylaminopurine (BAP) in concentrations of 1 mg/L and 3 mg/L, respectively [20]. The removal of the seedlings' roots stimulated sprouting of shoots from the stem section, without callus formation. The subsequent transfers to fresh medium with separation of shoots from each other enhanced shoot proliferation. However, *in vitro* plantlets turned pale after several weeks on this medium, in spite of the intensive light in the culture room. In the present study, we tested several media differing in the concentration of the minerals and the chosen PGRs, in order to examine their influence on the shoot multiplication effectiveness and the antioxidant activity of *A. mollis*, and also the possible relation between these parameters under the conditions of *in vitro* culture.

The cultures grown for two months on the four tested media differed significantly in their multiplication rates ($p<0.001$). The highest propagation coefficient (PC) was observed on the richest medium and the lowest one on the PGRs free medium (Figure 1). The cultures on the other two media had similar propagation rates; however, a more detailed investigation revealed essential difference in their regeneration potential (Figure 2).

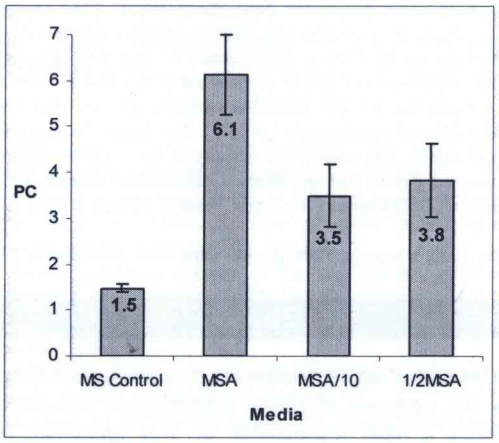


Figure 1: Propagation coefficient of *A. mollis* cultivated for 2 months on MS-based media with different composition: MS control – free of plant growth regulators; MSA – with 3 mg/L BAP and 1 mg/L NAA; MSA/10 – with 10-fold lower concentrations of BAP and NAA; ½MSA – half-strength macrosalts, with PGRs like MSA.

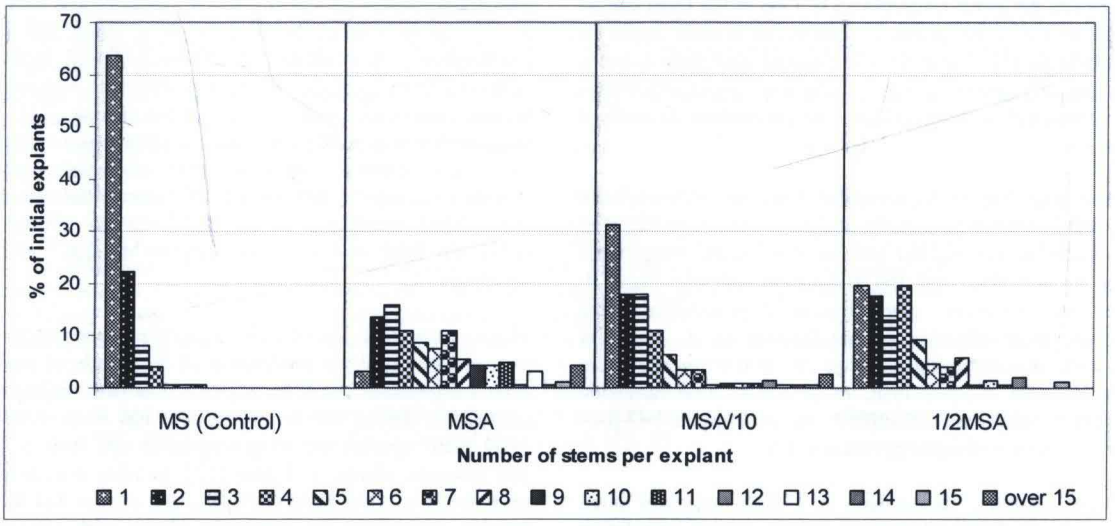


Figure 2: Sprouting capacity of uniform *A. mollis* shoots cultivated for two months on the four tested media (MS control, PGR-free; MSA, supplemented with 3 mg/L BAP and 1 mg/L NAA; MSA/10, supplemented with 0.3 mg/L BAP and 0.1 mg/L NAA; ½MSA, with half-strength macrosalts and PGRs like MSA).

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About one third (31.2%) of the shoots grown on medium MSA/10 (with 10-fold reduced PGRs compared with media MSA and ½MSA), did not form new shoots, and the number of the shoots regenerating only one or two new shoots was also high. In comparison, on the medium ½MSA the number of shoots remaining single was equal to that of the shoots regenerating three new shoots: 19.6% for each of these two groups. The total PC of the culture grown on medium MSA/10 was relatively high due to the fact that many shoots formed rhizomes at their base from which rose up numerous small new shoots, up to 29. The maximal number of shoots in the clusters formed on medium ½MSA was 15. On the richest medium (MSA), only 3.1% of the shoots remained single and 4.3% formed large clusters consisting of more than 15 shoots, reaching a maximum of 32. The single shoots on the PGR-free control medium MS represented 63.5% of all the initial shoots and the shoots regenerating more than 3 new ones were an exception. The detailed analysis of the data with respect to the distribution of the shoots after their sprouting capacity revealed that, in a way, medium MSA/10 was more similar to the control MS medium, while medium ½MSA resembled medium MSA.

In addition to the evident differences of the multiplication rates, plantlets cultivated on the different media differed in their morphological features (Figure 3). Those grown on the full-strength medium rich in PGRs (MSA) were smaller, with tiny pale green leaves, and did not form roots. In contrast, plantlets grown on the control MS medium were twice as high, with well expanded deep-green leaves, and with long roots having trichomes. Plantlets on the other two media had intermediate appearance; however, those grown on medium MSA/10 were more similar to the control ones concerning their height and leaf color, while those grown on medium ½MSA differed slightly from the plantlets cultured on medium MSA. Most of the plantlets on MSA/10 formed long roots; the rest, which formed large clusters, did not root. Only few plantlets on medium ½MSA rooted and these were short and lacked trichomes. It was remarkable that once rooted, plantlets grew bigger and their leaves turned dark-green in a short time.

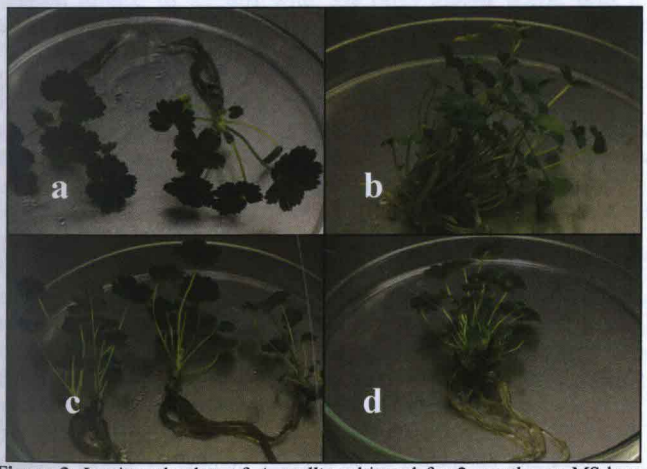


Figure 3: In vitro plantlets of *A. mollis* cultivated for 2 months on MS-based media with different composition: (a) MS control, free of PGRs; (b) MSA, supplemented with 3 mg/L BAP and 1 mg/L NAA; (c) MSA/10, supplemented with 0.3 mg/L BAP and 0.1 mg/L NAA; (d) ½MSA, with half-strength macrosalts and PGRs like MSA

The cultures grown on the four media differed also in the percentage of their dry matter (Table 1). Shoots grown on the media with high PGR concentrations were more affected; however, the decrease of the minerals in medium ½MSA compensated partially for this negative effect. Significant increase of the fresh weight/dry weight ratio indicating higher water content in the plant tissues was reported also for the Balkan endemic species *Hypericum rumeliacum* Boiss., cultured on BAP-supplemented medium [21]. Histological analysis of *Lamium album* L. proved the negative influence of exogenous BAP on the cell structures when it was applied solely in the medium, even in much lower concentration (0.2 mg/L) [22]. The authors reported that the leaf assimilative parenchyma was more affected than the epidermal tissue; the palisade cells shortened, became more rounded and coupled whereas large intracellular spaces formed between them. In comparison, the combination of auxin and cytokinin in low concentrations (0.2 mg/L BAP and 0.5 mg/L IBA) led to a regular structure of the photosynthetic tissues, which favored the stage of *ex vitro* adaptation of the plantlets.

In our experiment, the low dry matter and the pale green color of the leaves of the shoots grown on medium MSA were probably due to the disturbed assimilative parenchyma. Apparently, these structural changes were transient because after the transfer to PGR-free medium all plantlets rooted in a short period and restored the normal dark green color of their leaves. Similarly, comparative study of the leaf tissues of *in vitro* propagated and *ex vitro* adapted plants of the widely used medicinal plant of South East Asia - *Orthosiphon stamineus*, revealed that the average thickness of the leaf lamina and all its tissues (mesophyll, palisade and spongy parenchyma, adaxial and abaxial epidermis) decreased almost twice under *in vitro* conditions, but normalized after the *ex vitro* adaptation of the plants [23]. The histological organization of the photosynthetic apparatus of the *in vitro* plants was not affected and no considerable changes in the palisade factor values were observed.

The contents of flavonoids and tannins in the *in vitro* cultures of *A. mollis* cultivated on the four tested media differed significantly ($p<0.01$ and $p<0.05$, respectively) (Table 1). The highest flavonoid content was determined in the shoots cultivated on the control MS medium: 1.26±0.01% of the dry matter. It was commensurable with that in the *ex vitro* adapted plants on Vitosha Mt. The content of tannins was highest in the shoots grown on medium ½MSA.

Table 1: Features of *A. mollis* shoots grown *in vitro* on four nutrient media, and leaves of *ex vitro* adapted plants in Vitosha Mt. or one year old *in vivo* plants grown in Viotsha Mt. or Rhodopes Mts.: contents of dry matter, contents of flavonoids and tannins, and DPPH radical scavenging activity of the total methanolic extract.

Medium or Experimental plot	Dry matter (%)	Flavonoids (%)	Tannins (%)	IC_{50} µg/mL
MS Control	19.1	1.26±0.01 a	4.86±0.27 a	38.1±1.0 a
MSA	12.6	0.69±0.01 b	5.09±0.10 a	18.6±4.1 b
MSA/10	18.3	1.02±0.007 c	4.29±0.17 b	34.3±5.7 a
½MSA	16.0	0.98±0.009 d	6.23±0.08 c	19.1±2.2 b
Ex vitro, Vitosha	-	1.13±0.008 a	5.71±0.07 d	13.1±1.9 c
In vivo, Vitosha	-	0.78±0.01 b,e	4.22±0.09 e,b	27.5±7.0 d,b,a
In vivo, Rhodopes	-	0.80±0.008 e	4.50±0.10 f,b	22.2±6.0 d,b

Values with the same letter are not significantly different, $p \geq 0.05$. Standard deviations were calculated on the base of three samples.

Interestingly, the antioxidant activity of the *in vitro* cultures correlated with the concentrations of the PGRs in the respective media. The shoots cultivated on the media rich in BAP and NAA had much highest antioxidant activity than those on the media free of PGRs or with low concentrations of BAP and NAA ($p \leq 0.01$). Taking in consideration our preliminary results on cultivation of *A. mollis* in liquid MSA medium, with PC increased to 32.4±3.3

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shoots per explant for 6 weeks [20], this fact offers an opportunity to produce *in vitro* biomass of *A. mollis* as an alternative to its possible field cultivation. Shoot multiplication was highly effective in liquid medium MSA/10, as well reaching a PC of 33.8±6.8 shoots per explant for 8 weeks (unpublished data). Further determination of the secondary metabolites and the antioxidant activity of the *in vitro* produced biomass of *A. mollis* will elucidate the possible effect of the liquid cultures.

The *ex vitro* adapted plants on Vitosha Mt. were distinguished by the highest antioxidant activity, with an IC_{50} of 13.1±1.9 μg/mL. It is worthy of notice that the IC_{50} value of the commercial antioxidant, butylated hydroxytoluene (BHT), used as a positive control is 12.6 μg/mL. Compared with the one year old *in vivo A. mollis* plants cultivated on the mountains Vitosha and Rhodopes, the *in vitro* propagated and *ex vitro* acclimated to Vitosha Mt. plants had higher contents of flavonoids and tannins, and also much higher antioxidant activity. This could be due to the stress they had to overcome for a short time during the adaptation first to room and greenhouse conditions, and then to the ambience of 1500 m a. s. l. Further determination of the secondary metabolites in these plants during the next spring and their first flowering stage will be performed and the antioxidant activity of the plants derived from *in vitro* and *in vivo* propagation will be compared.

Shoots of *A. mollis* regenerated on the control MS medium had lower or closely similar DPPH antiradical scavenging capacity as the *in vivo* cultivated plants on Vitosha and Rhodopes mountains. Similarly, *in vitro* plantlets of *Lamium album* grown on PGR-free MS medium had approximately two times lower total phenols in comparison with the plants collected from nature [24]. These authors reported 5 to 7 times lower scavenging capacity in the *in vitro* extracts compared with the corresponding *in vivo* extracts. The total flavonoids and phenolics in the above ground parts of the Balkan endemic plant *Hypericum rumeliacum* collected from its natural habitat during the flowering period were slightly higher than those of the *in vitro* cultures grown on MS control medium [21]. In contrast, cultivation on medium supplemented with BAP led to an additional decrease in their levels; however, the retransfer to PGR-free MS medium caused regaining of these secondary metabolites, almost reaching the initial levels. The authors suggested as possible reasons for the negative effect of BAP on the biosynthetic capacity of *H. rumeliacum* shoots either the very intensive shoot proliferation or the predominance of stem tissue.

According to our results, the addition of PGRs in the nutrient medium was essential for the effective shoot multiplication of *Alchemilla mollis*. The medium MSA containing 3 mg/L BAP and 1 mg/L NAA ensured the highest propagation rate, which was further enhanced in liquid culture. Because of the high antioxidant activity of the shoots grown on the media supplemented with these two PGRs, it is promising to further test cultivation of *A. mollis* in liquid media and in a temporary immersion system, as an opportunity to develop a protocol for *in vitro* production of biomass of this species. In addition, *A. mollis* shoots can be multiplied on MSA medium and transferred to PGR-free MS medium for rooting and strengthening before *ex vitro* adaptation. Due to the ecological plasticity of the species, the *in vitro* produced plantlets could be acclimated at different altitudes, and field cultivated as an alternative to their collection from nature.

Experimental

Plant material: origin and samples for analyses: Rhizomes of *A. mollis* were collected in 1990 from its unique Bulgarian population situated in the Dzhendema Reserve (at 1162 m a. s. l.), which is a part of the Central Balkan National park, and were maintained in two *ex situ* collections - in the Sofia region and the Rhodopes Mts. A voucher specimen was deposited in the herbarium of the Institute of Botany, Sofia (SOM 159980). They were used for establishment of an *ex situ* collection in the experimental plot of the Institute of Botany in Sofia (about 500 m a. s. l.). The plants have been maintained for several years. In 2010, seeds were taken from them for *in vitro* culture initiation while rhizome cuttings were used for *in vivo* cultivation in the experimental areas located in the Rhodopes Mts. and Vitosha Mt., both at 1500 m a. s. l. One year later, the *in vitro* cultures consisted of thousands of well- shaped plantlets and clusters of plantlets with leaves and roots, while 80% of the *in vivo* plants developed 1 to 6 flower stems and formed clusters with projections of about 38 cm in diameter. In addition, some *in vitro* regenerated plantlets were adapted *ex vitro*, first in room conditions, in a mixture of soil, sand and coconut fiber (2:1:1), and then consecutively acclimated to greenhouse ambience and to the Vitosha Mt. experimental area. In September 2011, the projection of their rosettes was about 15 cm in diameter, developed for a period of 15 weeks after planting in Vitosha Mt. Samples representing either *in vitro* plantlets with removed roots, or leaves of *in vivo* or *ex vitro* adapted plants were analyzed for radical scavenging activity, flavonoid and tannin contents.

In vitro *cultivation:* Seeds were surface sterilized by consecutive treatment with 70% ethanol for 1 min and commercial bleach (chlorine < 5%) for 10 min, then rinsed 30 min with sterile distilled water. The medium for seed germination and for cultivation during the first nine months was MS-based [17], solidified with 6.5 g/L plant agar (Duchefa, NL) and supplemented with 30 g/L sucrose, 1 mg/L NAA and 3 mg/L BAP; pH was adjusted to 7.5 before autoclaving at 121°C for 20 min. Cultivation conditions were constant: 23±2°C, 16 h light daily with intensity of 3000 lux, in plastic containers (Vitro vent, Duchefa, NL). Seedlings' roots were removed during the first transfer to fresh medium; during the following transfers, every 3 months, plants were subcultured by separation of the new shoots grown from the stem base and by cutting of the biggest ones into 2 or 3 segments.

Uniform, separate *A. mollis* shoots of nine-month old *in vitro* cultures were used to study the effects of the medium composition (especially the concentration of the 2 plant growth regulators and that of the minerals) on the propagation rate, as well as on the biosynthetic activity of the plantlets. Four MS-based media were compared: the initial one, containing 1 mg/L NAA and 3 mg/L BAP (medium MSA), medium with 10-fold lower concentrations of NAA and BAP, i. e. 0.1 mg/L NAA and 0.3 mg/L BAP (medium MSA/10), medium with plant growth regulators like medium MSA, but with half-strength macrosalts and microelements (medium ½MSA), and control medium without any plant growth regulators (MS control). Shoots were grown for 2 months on these media, in plastic containers (28 shoots per Vitro vent container, Duchefa, NL), and then the multiplication rates of the cultures were compared by evaluation of the propagation coefficients (PC) for each medium, calculated as an average number of shoots obtained per explant. Differences concerning the multiplication rate efficiency were assessed by ANOVA single factor analysis based on 7 repetitions of 28 shoots each, grown on each of the media.

DPPH radical scavenging activity: The effect of the methanolic extracts of the studied samples on DPPH radical was estimated according to Choi *et al.* [25] and Stanojević *et al.* [26]. Different concentrations of plant extract (5, 10, 20, 50, 100 and 200 μg/mL), in methanol, were added at an equal volume (2.5 mL) to a methanol solution of DPPH (0.3 mM, 1.0 mL). After 30 min at room

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temperature, the Ab values were measured at 517 nm on a spectrophotometer (Jenway 6320D) and converted into the percentage antioxidant activity using the following equation: DPPH antiradical scavenging capacity: (%) = [1-(A_{sample} - A_{blank})/$A_{control}$] x 100. Methanol (1.0 mL) with plant extract solution (2.5 mL) was used as a blank, while DPPH solution with methanol was used as a control. The IC_{50} values were calculated by a sigmoid non-linear regression model using plots, where the abscissa represented the concentration of tested plant extracts, and the ordinate - the average percent of scavenging capacity (Prizm 3.00 software). IC_{50} values denote the concentration of sample required to scavenge 50% of the DPPH radicals. The commercial antioxidant butylated hydroxytoluene (BHT) and syringic acid were used as positive controls and their IC_{50} values were respectively 12.6 and 4.4 µg/mL. All determinations were performed in triplicate (n = 3). Differences between the extracts were assessed by ANOVA single factor analysis.

Determination of flavonoids: The determination of flavonoid content in the investigated extracts was performed spectrophotometrically at 425 nm by formation of a complex with $AlCl_3$ according to the European Pharmacopoeia, with slight modification [27]. Briefly, acetone (20 mL) and 36% HCl (2 mL) were added to the dried and ground sample of leaves or *in vitro* shoots (0.15-0.20 g) and boiled under reflux for 30 min. After filtration, the residue was re-extracted twice with acetone (20 mL) for 10 min and the volume of filtrates was made up to 100 mL with acetone. An aliquot (20 mL) of the acetone extract was put into a separation funnel, along with water (20 mL) and ethyl acetate (15 mL). The extraction with ethyl acetate was carried out 3 times and the combined ethyl acetate layers were then washed twice, using 20 mL of water each time, and were subsequently diluted to 50 mL with ethyl acetate. To 10 mL of this solution, in a volumetric flask were added 1 mL 2% (w/v) $AlCl_3$ solution in 5% (v/v) glacial acetic acid in methanol and the mixture was made up to 25 mL with 5% (v/v) glacial acetic acid in methanol. After 30 min, the absorbance was measured at 425 nm against the blank (10 mL of sample solution diluted to 25 mL with 5% (v/v) glacial acetic acid in methanol). The flavonoid content was calculated using quercetin as a standard, and the results were expressed as g quercetin equivalents per 100 g of dry plant material. All determinations were performed in triplicate (n = 3). Differences between the extracts were assessed by ANOVA single factor analysis.

Determination of tannins: Determination of total tannins was performed following the method described in the European Pharmacopoeia [28]. Briefly, the dried and ground leaves or *in vitro* shoots (0.20 -0.25 g) were heated for 30 min in a water bath with water (150 mL), then transferred to a 250 mL volumetric flask and made up to 250 mL with water. The obtained solution was filtered and the first 50 mL of the filtrate was discarded; 5 mL of the remaining filtrate was diluted to 25 mL with water and served as a stock solution. An aliquot of stock solution (2 mL) was mixed with Folin–Ciocalteu's phenol reagent (1 mL) and 10 mL water, and diluted to 25 mL with sodium carbonate solution (29% $Na_2CO_3.10H_2O$). After 30 min, the absorbance was read at 760 nm (A_1). Hide powder (0.1 g) was added to 10 mL of the stock solution and vigorously shaken for 60 min. After filtration, 5 mL of the resulting filtrate was diluted with water in a volumetric flask up to 25 mL. An aliquot of the latter (2 mL) was mixed with Folin–Ciocalteu's phenol reagent (1 mL) and 10 mL of water, and diluted to 25 mL with sodium carbonate solution (29% $Na_2CO_3.10H_2O$). After 30 min, the absorbance was read at 760 nm (A_2). The percentage content of tannins, expressed as pyrogallol, was calculated from the following equation: (%) = $3.125.(A_1-A_2)/(A_3.m)$, where A_1, and A_2 were the measured absorbances of the sample solutions before and after hide powder treatment, respectively, A_3 – the absorbance of the test solution containing 0.05 g of pyrogallol, and m – the mass of the dry plant material (g). All determinations were performed in triplicate (n = 3). Differences between the extracts were assessed by ANOVA single factor analysis.

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