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Antioxidant Capacity and Accumulation of Caffeoylquinic Acids in *Arnica montana* L. In Vitro Shoots After Elicitation with Yeast Extract or Salicylic Acid

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Abstract: *Arnica montana* L. is an important herbal medicinal plant that belongs to the family Asteraceae. This plant has been known for its medicinal uses for centuries. *A. montana* exhibits several pharmacological properties, including immunomodulatory, anti-inflammatory, anticancer, antioxidant, and antibacterial effects. For the first time, the impacts of the biotic elicitor yeast extract, and the abiotic elicitor salicylic acid on micropropagation, antioxidant potential, and accumulation of caffeoylquinic acids in arnica in vitro shoots were assessed. The results showed that yeast extract applied at 100 mg/L significantly promotes shoot multiplication, biomass yield, total phenolic content, and synthesis of caffeoylquinic acids compared to control untreated shoots. Flavonoid content was the highest in samples treated with 200 mg/L of yeast extract, although at this concentration the measured biometric parameters began to decrease. Salicylic acid at 100 µM was found to be effective in the induction of vigorous shoots, shoot height growth, and biomass accumulation; nevertheless, this elicitor downregulated the caffeoylquinic acid level, total phenolics, and flavonoids. Increasing the concentration of salicylic acid to 200 µM caused shoot multiplication and fresh biomass accumulation reduction. Both elicitors modulated the activity of antioxidant enzymes against oxidative stress. Overall, the use of these substances can improve the growth and biomass yield in *Arnica* in vitro shoots.

Keywords: micropropagation; total phenolic content; total flavonoid content; caffeoylquinic acids; HPLC; antioxidant potential

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1. Introduction

Arnica montana L., also known as mountain tobacco, is a medicinal plant that has been used for centuries in European medicine. The plant species belongs to the Asteraceae family and generally grows in nutrient-poor and dry heathlands, shrublands, and grasslands of mountains. *A. montana* is a source of more than 150 biologically active compounds, the majority of which were classified as phenolic compounds (phenolic acids, flavonoids,

a basis for future research into the increase in phenolic compounds with strong antioxidant activity in this highly valued medicinal plant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants14060967/s1, Figure S1: The structures of the main compounds identified in *A. montana* shoots.

Author Contributions: M.P. and M.S. (Magdalena Sozoniuk), conceptualized the research; M.P., K.M.-G., L.D. and M.D., micropropagated and treated the in vitro plants; M.G., A.T., K.M.-G., M.S. (Mariana Sichanova), V.I. and M.N. performed the laboratory analyses; M.P., K.M.-G., M.G. and A.T., prepared figures and photos; M.P. and K.M.-G., prepared the original draft of the manuscript; M.S. (Magdalena Sozoniuk), M.G. and A.T., reviewed and edited the manuscript; M.P. administrated the project. All authors have read and agreed to the published version of the manuscript.

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RESEARCH

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Identification and validation of reference genes with stable expression under elicitor treatments of the medicinal plant *Arnica montana* L.

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Abstract

Background In view of enhancing secondary metabolites biosynthesis in *Arnica montana* through elicitation, comprehensive studies are needed to fully understand the molecular background of biosynthetic pathways in this species. Analysis of transcriptional changes via RT-qPCR technique might shed light on the molecular mechanisms underlying plant reaction to elicitors. This study aimed to identify reference genes which are stably expressed in *Arnica* under methyl jasmonate, salicylic acid, and yeast extract treatment to provide the basis for current and future gene expression studies in this important medicinal plant.

Results The expression stability of nine candidate reference genes was evaluated using four widely used algorithms (geNorm, NormFinder, BestKeeper, and ΔCt method). A comprehensive analysis of the obtained results showed that the most stably expressed pair of genes under elicitation conditions was *ATP-synthase* and *ACT*. The *PP2 A* and *TUBb* were the pair of least stable candidates as they presented substantial variation in transcript levels in response to elicitor agents. For validation purposes, the transcriptional profile of *PAL*, *4 CL* and *HQT* genes was analyzed. Substantial induction of two of these biosynthetic genes was confirmed after methyl jasmonate treatment.

Conclusions The *ATP-synthase* in combination with *ACT* were identified as the best endogenous controls for RT-qPCR data normalization in elicitation studies of *A. montana*. The research outcomes shed light on transcriptional changes associated with arnica's response to elicitation and contribute to the understanding of secondary metabolism regulation in medicinal plants.

Keywords Reference genes, Arnica, Gene expression, Elicitation, Methyl jasmonate, Secondary metabolites

Introduction

Arnica montana L. is a valuable medicinal plant belonging to the Asteraceae family. The herb has been used in European traditional medicine for many centuries. The species is especially rich in biologically active compounds such as sesquiterpene lactones, flavonoids, fatty acids, thymol derivatives, and chlorogenic acid [1]. *Arnica* metabolites possess anti-inflammatory, anticancer, antioxidant, antimicrobial, antiplatelet, and immunomodulatory properties. *A. montana* is a rare and endangered species due to over-collection for herbal use, loss of

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Lilium brownii [30] or *Arachis hypogaea* [31], we found it to be the best-performing RG in arnica tissue cultures under elicitation. Three out of four used algorithms (geNorm, BestKeeper, and ΔCt method) unanimously ranked *ATP-synthase* as one of two the most-stable RGs in the tested material. Only NormFinder positioned it as the fourth RG in a stability ranking, which still indicates low expression variability. Even though *ATP-synthase* is not as commonly used as other traditional RGs, such as *GAPDH*, *ACT*, *EF1*a, or α*-/β-TUB* [32–34], our results suggest that it might be a worthy candidate for data normalization in elicitation research.

Our previous study focused on monitoring expression profiles of candidate RGs during different stages of *A. montana* and *Arnica chamissonis* flower development [17]. We found *SKIP16* and *F-box* to be the most stably expressed RGs in the developing flowers of *A. montana*. In the cross-species dataset also *F-box,* but in combination with *ACT,* was recommended as the best internal control. Here, *F-box* ranked as third RG, which also indicates its good expression stability and its suitability as a candidate endogenous control in future gene expression research.

For validation purposes of selected RGs, we chose three target genes involved in phenolic compound metabolism. Both *PAL* and *4 CL* belong to the early biosynthetic genes. They encode phenylalanine ammonia lyase and 4-coumarate:CoA ligase, respectively, and are involved in the core phenylpropanoid pathway. The last one, *HQT*, encodes hydroxycinnamoyl-CoA:quinic acid hydroxycinnamoyltransferase, represents late biosynthetic genes and is a key enzyme involved in the chlorogenic acids pathway [35, 36]. Expression of abovementioned genes is known to be influenced by different factors, such as environmental conditions, pathogen attack, or phytohormones [37–39]. Here we showed that the expression of both *PAL* and *HQT* was upregulated by MeJA treatment, regardless of the elicitor concentration used. This is congruent with other reports concerning various plant species. For instance, *PAL* was found to be upregulated by MeJA treatment in *Castilleja tenuifora* [40], *Isatis indigotica* [41], *Ocimum tenuiflorum* [42] or *Ocimum basilicum* [43]. The *HQT* was induced in response to MeJA treatment in *C. intybus* [44], *Solanum tuberosum* [45] and *Ipomoea batatas* [46]. On the other hand, our research showed that *4 CL* gene was unresponsive to the treatment with any of the elicitors.

Previous studies have demonstrated that *HQT* plays a major role in the chlorogenic acids (CGAs) production as a rate-limiting step in their biosynthesis pathway [39, 47, 48]. For instance, overexpression of *HQT* resulted in the accumulation of CGAs in *Taraxacum antungense*, while *HQT* suppression decreased their level [49]. In this study, increased transcript levels of target genes corresponded with our results of quantification of mono-, di- and tri-caffeoyl quinic acids. The observed upregulation of *PAL* and *HQT* caused by MeJA treatment was accompanied by increased accumulation of these compounds (unpublished data).

Conclusions

The expression stability investigation of a set of nine candidate RGs in *A. montana* subjected to elicitation identified *ATP-synthase* in combination with *ACT* as the best endogenous controls for RT-qPCR data normalization. Moreover, *PP2 A* and *TUBb* were found to display substantial expression variability in response to elicitor agents, hence they are not recommended to be used as internal controls in elicitation studies. These findings provide helpful information for future research on the molecular processes regulating secondary metabolite biosynthesis in medicinal plants.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06557-z.

Additional File 1: Tab. S1. Sequences and characteristics of primers designed for candidate reference genes and genes of interest. Fig. S1. Melting curves obtained from analysis of candidate reference genes

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The authors comply with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

Authors' contributions

MS and MP conceptualized the research, MP, KM-G micropropagated and treated the in vitro plants, MS and KM performed the laboratory analyses, MS analyzed the data and prepared original draft of manuscript, MP wrote part of the manuscript, MP, KM, KM-G, MG reviewed and edited the manuscript, MP administrated the project. All authors have read and agreed to the published version of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

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Improvement of *Stevia rebaudiana* Bertoni *In Vitro* Propagation and Steviol Glycoside Content Using Aminoacid Silver Nanofibers

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Abstract: The food industry is interested in replacing artificial sweeteners with natural sugars that possess zero calories and carbohydrates and do not cause spikes in blood sugar levels. The steviosides leaves, synthesized at *Stevia rebaudiana* Bertoni, are 300 times sweeter than common table sugar. *Stevia* propagation is limited due to the poor viability of the seeds, the long time and low germination rate, and the poor rooting ability of vegetative cuttings. Because of this, an alternative biotechnological method for its reproduction is being studied, such as multiple shoot production through direct organogenesis using nanofibers, formed from a derivative of amino acid valine as a carrier of the biologically active agent silver atoms/particles (NF-1%Ag and NF-2%Ag). The stevia explants were cultured on a medium containing NF-1%Ag and NF-2%Ag at concentrations of 1, 10, 50, and 100 mg L^{-1}. The NF-1%Ag and NF-2%Ag treatment caused hormetic effects on stevia plantlets. At low concentrations of from 1 to 50 mg L^{-1} of nanofibers, the stimulation of plant growth was observed, with the maximum effect being observed at 50 mg L^{-1} nanofibers. However, at the higher dose of 100 mg L^{-1}, inhibition of the values of parameters characterizing plant growth was recorded. The presence of nanofibers in the medium stimulates stevia root formatting.

Keywords: antioxidant activity; *in vitro* propagation; nanofibers; carrier of Ag particles; *Stevia rebaudiana* Bert.

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1. Introduction

Since ancient times, people have mainly used plants to treat various diseases. Pharmacy has created modern contemporary medicines by studying the biologically active properties of secondary metabolites in plants. The production of a large percentage of current therapeutic agents is based on the use of natural products derived from medicinal and aromatic plants. Therefore, it is necessary to cultivate medicinal and aromatic plants in conditions under which certain biologically active secondary metabolites necessary for the pharmaceutical, cosmetic, and food industries will be synthesized.

Stevia (*Stevia rebaudiana* Bertoni) [1] is a valuable medicinal plant of the Asteraceae family with wide application in the pharmaceutical and food industry. The food industry is increasingly interested in replacing artificial sweeteners with other natural sugars in order to offer the consumer a wider range of choices and to satisfy the requirements of a segment of the population that does not want to or cannot eat sucrose. The stevia leaves have been used as a low-calorie sweetener for centuries, and are currently consumed worldwide. The sweetness of this plant is due to the accumulation of bioactive compounds, especially

the *in vitro* growth and production of stevia, offering the possibility for its introduction into agriculture.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11192468/s1. Figure S1: *S. rebaudiana* plantlets *in vitro* propagated on MS medium (A control plants); MS medium containing 0.5 mg L^{-1} BAP (B); MS medium with various concentrations (1, 10, 50, 100 mg L^{-1}) of aminoacid nanofibers enriched with 1% (C, D, E, F) and 2% colloidal (G, H, I, J) Ag particles (NF-1%Ag, NF-2%Ag).

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Multiplication and Conservation of Threatened Medicinal Plant *Arnica montana* L. by *in vitro* Techniques

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Summary

An efficient and reproducible *in vitro* protocol for mass production of the threatened medicinal plant *Arnica montana* L. (Asteraceae) was developed. The effectiveness of various combinations of plant growth regulators on *A. montana* clonal multiplication was assessed, using seedlings' stems as initial explants. Among 12 tested nutrient media, the optimum one (MS supplemented with 1.0 mg/l BAP and 0.1 mg/l IAA) increased the organogenesis frequency up to 95% in the best origin, with mean number of shoots per explant 4.25 for 5 weeks. Sub-cultivations on this medium every 4 weeks led to increase of the propagation rate as in the fifth subculture the average number of shoots per explant reached 12.32±0.82. Rooting of uniform *in vitro* shoots was 100% successful on half strength MS medium supplemented with 0.5 mg/l IBA. The *ex vitro* adapted plants showed 90% survival, and were further acclimatized to two mountain *ex situ* collections. Plants looked healthy and true-to-type and began to bloom in the second or the third year. In addition, a successful protocol for slow-growth storage of *in vitro A. montana* cultures was elaborated, after testing 8 media with mannitol or sorbitol. The medium ½ MS containing 3% sorbitol and 2% sucrose was chosen as the best one, efficiently retarding the growth of the *in vitro* plantlets, thus allowing 6-month maintenance without sub-cultivation. The developed *in vitro* protocols could be of great value for commercial propagation and sustainable conservation of this threatened medicinal plant.

Key words

in vitro culture, plant growth regulators, multiplied shoots, slow-growth storage

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Antioxidant activity of in vitro propagated *Stevia rebaudiana* Bertoni plants of different origins

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Abstract: An efficient in vitro protocol for propagation of *Stevia rebaudiana* Bertoni is described. Multiple shoots were induced in vitro from shoot tip and nodal segments on Murashige and Skoog medium containing 6-benzylaminopurine, zeatin, or thidiazuron alone and in combination with naphthalene acetic acid or indole-3-acetic acid. A high frequency of shoot induction as well as maximum number of shoots per shoot tip explant was observed on Murashige and Skoog medium supplemented with 6-benzylaminopurine (1.0 mg L^{-1}) alone and combined with indole-3-acetic acid (0.1 mg L^{-1}). For root induction, in vitro shoots were transferred to rooting media containing naphthalene acetic acid, indole-3-acetic acid, or indole-3-butyric acid. The highest rooting frequency and the highest number of roots was observed in half-strength Murashige and Skoog medium supplemented with 0.1 mg L^{-1} indole-3-butyric acid. The rooted in vitro plants were successfully acclimatized in a growth chamber and transferred to the field. Leaf extracts of plants propagated in vitro and adapted to field conditions are characterized by high levels of water-soluble antioxidant capacity (expressed as equivalents of ascorbic acid), phenols, and flavonoids, and therefore by high total antioxidant potential, expressed as DPPH radical scavenging activity.

Key words: Acclimatization, micropropagation, nodal segments, shoot tips

1. Introduction

Stevia rebaudiana Bertoni, belonging to the family *Asteraceae*, is a perennial sweet herb. It is a native medicinal plant of Paraguay and is a new alternative source of calorie-free sweetener having no carbohydrates. The leaves of this plant produce diterpene glycosides (stevioside and rebaudiosides). Pure stevioside is 30 times sweeter than sugar (1–4). Recently, food-derived antioxidants, such as vitamins and phenolic phytochemicals, have received growing attention because they are known to function as chemopreventive agents against oxidative damage (5). The dry extract from the leaves also contains flavonoids, alkaloids, water-soluble chlorophylls and xanthophylls, hydroxycinnamic acids (caffeic, chlorogenic, etc.), neutral water-soluble oligosaccharides, free sugars, amino acids, lipids, essential oils, and trace elements (6). Plants constitute an important source of active natural products, which differ widely in terms of structure, biological properties, and ways of propagation. Therefore, it is of great interest to evaluate the nonenzymatic antioxidants and the water-soluble and lipid-soluble antioxidant capacities (expressed as equivalents of ascorbate and α-tocopherol), total phenolic compounds, flavonoids, and free radical scavenging activity of *Stevia rebaudiana* Bertoni propagated in different ways. Although phenolic compounds do not have any nutritional function, they may be important to human health because of their antioxidant potential (7). Therefore, the study of the importance and role of nonnutrient compounds, particularly phenolic acids, flavonoids, and high molecular tannins, as natural antioxidants has greatly increased (8). Natural antioxidants such as α-tocopherol and ascorbic acid are widely used because of their free radical scavenging activity (9). The leaf extract of the stevia plant has been used in the treatment of diabetes (10). It also enhances weight reduction, prevents dental caries, and has antimicrobial properties. It is reported that *S. rebaudiana* Bertoni also contains an antioxidant, steviol (11,12).

This species can be propagated by seed, by vegetative cutting, and by tissue culture. Seed germination is very poor, commonly due to infertile seed (13). Vegetative propagation by stem cutting is limited and requires enough stocks of stem cuttings (14,15). Thus, the development of an efficient alternative method for mass micropropagation of *S. rebaudiana* Bertoni is important for large-scale plant production. A number of protocols for in vitro propagation of this species have been described during recent years (16–23). According to Cenkci et al. (24), both

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Morphological evaluation and antioxidant activity of *in vitro*- and *in vivo*-derived *Echinacea purpurea* plants

Research Article

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Abstract: An effective *in vitro* protocol for rapid clonal propagation of *Echinacea purpurea* (L.) Moench through tissue culture was described. The *in vitro* propagation procedure consisted of four stages: 1) an initial stage - obtaining seedlings on Murashige and Skoog (MS) basal medium with 0.1 mg L^{-1} 6-benzylaminopurine, 0.1 mg L^{-1} α-naphthalene acetic acid and 0.2 mg L^{-1} gibberellic acid; 2) a propagation stage - shoot formation on MS medium supplemented with 1 mg L-1 6-benzylaminopurine alone resulted in 9.8 shoots per explant and in combination with 0.1 mg L^{-1} α-naphthalene acetic acid resulted in 16.2 shoots per explant; 3) rooting stage - shoot rooting on half strength MS medium with 0.1 mg L^{-1} indole-3-butyric acid resulted in 90% rooted microplants; 4) *ex vitro* acclimatization of plants. The mix of peat and perlite was the most suitable planting substrate for hardening and ensured high survival frequency of propagated plants. Significant higher levels were observed regarding water-soluble and lipid-soluble antioxidant capacities (expressed as equivalents of ascorbate and α-tocopherol) and total pnenols content in extracts of *Echinaceae* flowers derived from *in vitro* propagated plants and adapted to field conditions in comparison with traditionally cultivated plants.

Keywords: *Purple coneflower • In vitro shoots • Seedlings • Morphological traits • Antioxidant activity*

Abbreviations

MS - Murashige and Skoog medium;
PGR - plant growth regulator;
BAP - 6-benzylaminopurine;
GA_3 - gibberellic acid;
AA - ascorbic acid;
IBA - indole-3-butyric acid;
NAA - α-naphthalene acetic acid.

1. Introduction

Echinacea purpurea L. Moench (Asteraceae) or purple coneflower is a widespread medicinal plant used in diverse range of herbal products. It was proved that Echinacea is one of the most promising immune strengtheners and modulators, with numerous scientific studies and rich clinical evidence in its favor [1-3]. The increasing demand in *E. purpurea* needed development of methods for rapid multiplication of plants and faster introduction of new cultivars with desired traits [4]. However, *E. purpurea* plants produced highly heterozygous progeny in the field [5]. In this regard, *in vitro* tissue cultures are proved to be valuable technique to produce genetically homogeneous plant material. There were identified 58 unique germplasm lines based on screening for antioxidant activity and concentrations of caftaric acid, chlorogenic acid, cichoric acid, cynarin, and echinacoside from clonal propagated seedling-derived plants [6].

Several *in vitro* techniques were developed in *E. purpurea* [3,4,7-9] as some of the genotypes showed high coefficient of *in vitro* propagation [8,10,11]. The application of biotechnological techniques might offer the possibility of producing large amount of uniform high-quality plants in a short period of time and limited space for obtaining a biomass as a source of biological active compounds [3]. Nevertheless, many questions about its *in vivo* and *in vitro* culture remain still unsolved. In Bulgaria, *E. purpurea* is grown on limited area and the information

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Article

Effect of In Vitro Pretreatment with Ag-Containing Amino Acid Nanofibers on Biometrics and Antioxidant Activity in Drought-Stressed Ex Vitro-Adapted *Stevia rebaudiana* Bertoni

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Abstract: Biotechnological methods prevent the destruction of natural populations of medicinal plants due to climate change and developing agriculture. This study evaluates the effects of in vitro pretreatment with two types of silver-containing amino acid nanofibers (NF-1%Ag and NF1-Ag salt) on the drought tolerance of ex vitro soil-adapted *Steviia rebaudiana* Bertoni. The duration of the drought was five days. The data suggested that the pretreatment with the studied nanofibers during plant propagation enhanced the plant tolerance to drought stress manifested in a smaller decrease in plant biomass accumulation and a smaller increase in sugar content. The pretreatment with the two tested nanoparticles of well-watered plants increased the leaf fresh biomass accumulation of the ex vitro-adapted *S. rebaudiana* compared to the untreated WW control plants. The highest values were reported at 10 mg L^{-1} NF1-Ag salt. Five days of drought led to a decrease in the leaf fresh biomass compared to the WW plants, with the recorded lowest reduction again at 10 mg L^{-1} NF1-Ag salt. These observations correlate with antioxidant activity improvement. The results show that adding 10 mg L^{-1} NF1-Ag salt to the MS medium led to higher ex vitro-adapted *S. rebaudiana* resistance to water deficit than 100 mg L^{-1}. This paper discusses the impact of the selected nanofibers on parameters characterizing plant growth and antioxidant activity of drought-stressed ex vitro-adapted *Stevia rebaudiana* plants.

Keywords: *Stevia rebaudiana* Bert.; drought; biomass accumulation; antioxidant activity

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1. Introduction

Soil water reduction is one of the main environmental stress factors restricting plant growth [1,2]. Understanding how plants respond to drought stress (DS) can play an important role in improving crop management [3]. In response to a soil water shortage, plants have been identified to utilize several strategies to resist [3]. Among the fastest processes induced by drought is the abscisic acid-mediated closure of stomata [4] which reduces water loss due to transpiration. Prolonged DS leads to further acclimation responses including osmotic adjustment [5,6], a decreased shoot–root ratio [7], cell wall modifications [8], metabolism reorganization [9], and the activation of the antioxidant defense system [10].

Among the key strategies of plant drought tolerance are the regulation of antioxidant enzyme activity and the enhancement of antioxidant metabolite production [11]. Drought provokes stomatal closure and thus reduces gas diffusion. This, coupled with the ongoing photosynthesis in the light, results in the depletion of intercellular carbon dioxide. Reduced CO_2 availability stimulates ribulose-1,5-bisphosphate oxygenation and hence the photorespiratory production of hydrogen peroxide (H_2O_2) [12]. Plant cells produce oxygen

the MS nutrient solution, WS-AOM, LS-AOM, total phenols, and flavonoids are higher than in variants treated with 10 mg L^{-1} NF-1%Ag. The pretreatment with 10 mg L^{-1} NF1-Ag salt of ex vitro-adapted *S. rebaudiana* led to at least an increase, resulting from the drought stress, in the levels of hydrogen peroxide, proline, and malondialdehyde, and the most significant increase in the levels of sulfhydryl groups. This is accompanied by the most significant increase in water- and lipid-soluble metabolites with antioxidant potential, plant height, and leaf and stem dry biomass accumulation. At the higher concentration of 100 mg L^{-1} NF1-Ag salt, the largest extent of biomass reduction, the most significant increase in the level of the H_2O_2, MDA, and proline, and the most significant decrease in the level of the phenols, flavonoids, WS-AOM, LS-AOM, and SH group content were recorded, which is an indicator of the high oxidation stress. Consequently, the best mitigating effect of the harmful action of drought on plants was recorded when ex vitro-adapted *Stevia rebaudiana* plants were pretreated with 10 mg L^{-1} NF1-Ag salt. Future research must confirm this and identify the molecular function of these amino acid nanofibers under drought. Understanding the molecular mechanisms of how pretreatment with amino acid nanofiber carriers of silver impacts plants exposed to drought on growth and antioxidant activity is of crucial importance. For a short time of plant treatment, a large number of studies demonstrated the impacts of Ag-containing NPs on plants in various techniques, including morphological, physiological, cellular, and molecular levels. However, there is a lack of research about their impact after a long period (2 months after plant treatment). The findings of this study are based on controlled laboratory experiments, which are probably very different from field conditions. As a result, it is challenging to determine if the mechanisms of plant NF pretreatment and tolerance in the lab are identical to those in the field. Therefore, it is difficult to make a general conclusion on how the pretreatment with amino acid nanofiber carriers of silver, during in vitro propagation, responds to enhanced ex vitro-adapted *Stevia rebaudiana* drought tolerance after two months. Further research is required to evaluate the molecular pathways by which nanofibers influence plant drought resistance. Moreover, most of the research conducted over the past decade has concentrated on the effects of nanofibers on plants at morphological and physiological levels; however, the understanding of the effects at the molecular level requires further investigation to analyze the molecular mechanisms of amino acid Ag NPs and the tolerance mechanisms in plants.

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Review

Influence of Abiotic and Biotic Elicitors on Organogenesis, Biomass Accumulation, and Production of Key Secondary Metabolites in Asteraceae Plants

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Abstract: The medicinal plants of the Asteraceae family are a valuable source of bioactive secondary metabolites, including polyphenols, phenolic acids, flavonoids, acetylenes, sesquiterpene lactones, triterpenes, etc. Under stressful conditions, the plants develop these secondary substances to carry out physiological tasks in plant cells. Secondary Asteraceae metabolites that are of the greatest interest to consumers are artemisinin (an anti-malarial drug from *Artemisia annua* L.—sweet wormwood), steviol glycosides (an intense sweetener from *Stevia rebaudiana* Bert.—stevia), caffeic acid derivatives (with a broad spectrum of biological activities synthesized from *Echinacea purpurea* (L.) Moench—echinacea and *Cichorium intybus* L.—chicory), helenalin and dihydrohelenalin (anti-inflammatory drug from *Arnica montana* L.—mountain arnica), parthenolide ("medieval aspirin" from *Tanacetum parthenium* (L.) Sch.Bip.—feverfew), and silymarin (liver-protective medicine from *Silybum marianum* (L.) Gaertn.—milk thistle). The necessity to enhance secondary metabolite synthesis has arisen due to the widespread use of these metabolites in numerous industrial sectors. Elicitation is an effective strategy to enhance the production of secondary metabolites in in vitro cultures. Suitable technological platforms for the production of phytochemicals are cell suspension, shoots, and hairy root cultures. Numerous reports describe an enhanced accumulation of desired metabolites after the application of various abiotic and biotic elicitors. Elicitors induce transcriptional changes in biosynthetic genes, leading to the metabolic reprogramming of secondary metabolism and clarifying the mechanism of the synthesis of bioactive compounds. This review summarizes biotechnological investigations concerning the biosynthesis of medicinally essential metabolites in plants of the Asteraceae family after various elicitor treatments.

Keywords: in vitro culture; hairy roots; shoot culture; cell suspension; secondary metabolites; biosynthetic genes; elicitation

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1. Medicinal Plants from Asteraceae Family: Chemical Constituents and Applications

The Asteraceae family is one of the largest families of flowering plants in the world, with over 1600 genera and about 32,000 species [1]. Most of the Asteraceae family members have been used in medicine for centuries because of their various therapeutic applications. The plant species of Asteraceae contain a wide range of biologically active compounds; major among them are phenolic acids, flavonoids, terpenoids, volatile components, acetylenes, etc. [2]. They exhibit strong antibacterial, anti-inflammatory, antioxidant, anticancer, and antiparasitic activities, as well as diuretic and wound-healing qualities [3]. Several well-known species, such as *Artemisia annua* (sweet wormwood), *Stevia rebaudiana* (stevia), *Echinacea purpurea* (echinacea), *Chicorium intybus* (chicory), *Arnica montana* (mountain tobacco), *Tanacetum parthenium* (feverfew), and *Silybum marianum* (milk thistle), are of great interest to scientists because of their multispectral healing effects and wide uses in medicine, functional food, and cosmetic products.

to be the most significant tool in clarifying the synthesis of desired secondary metabolites in Asteraceae medicinal plants. More future studies are needed to establish elicitor-induced changes that lead to the upregulation of defense-related genes or the downregulation of non-defense-related genes, the transient phosphorylation or dephosphorylation of proteins, and the expression of key enzymes whose information can be used to determine the biosynthetic pathways of Asteraceae secondary metabolites. To gain a better understanding of the regulatory mechanisms controlling secondary metabolism, it is crucial to integrate genomics, transcriptomics, proteomics, and metabolomics. This could lead to the effective metabolic engineering of medicinally essential metabolites in plant in vitro systems.

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IN VITRO MULTIPLICATION AND GC/MS-BASED METABOLIC PROFILES OF *CICHORIUM INTYBUS* L.

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ABSTRACT

Cichorium intybus L. (Asteraceae) is one of the most widely used medicinal plants globally. The plant species is of great economic interest due to its high content of secondary metabolites. The present study was performed to compare the GC/MS-based metabolic profiles and total phenolic content of micropropagated and wild-growing plants. An optimized protocol for *in vitro* multiplication of *C. intybus* using stem segments from *in vitro* raised seedlings was developed. The optimum nutrient media were found to be MS medium supplemented with 1 mg/L BAP and 0.1 mg/L NAA and MS medium fortified with 1 mg/L 4PU-30 and 0.1 mg/L NAA, giving an average of 9.2±0.47 and 7.1±0.41 shoots per explant, respectively. The phenylurea cytokinin 4PU-30, first used for chicory micropropagation, effectively promoted plant regeneration and prevented hyperhydricity in *in vitro* plant tissue. Microshoots rooted successfully in half-strength MS medium free of plant growth regulators. All plants were hardened and survived transfer to *ex vitro* conditions. No differences were found between the GC/MS-based metabolic profiles of the wild-growing plants and those multiplied *in vitro* and acclimated to controlled field conditions. A quantitative difference was obtained in some individual metabolites: esculetin and quinic acid were higher in samples of *in vitro* obtained plants, while chlorogenic acid was more abundant in samples of wild-growing plants.

Keywords: micropropagation, callus, total phenols, metabolites, medicinal plant

INTRODUCTION

Cichorium intybus L. (Asteraceae) has a long history of use as a medicinal plant dating from ancient times. The species commonly known as chicory is one of the most popular medicinal plants on the global market and is also applied as a coffee substitute, vegetable crop and animal forage. Chicory is a unique plant species extremely rich in secondary metabolites – alkaloids, coumarins, sesquiterpene lactones, flavonoids, terpenoids, steroids, volatile compounds, phenolic acids, organic acids, caffeic acid derivatives (**Nandagopal and Kumari, 2007; Aisa *et al.* 2020**). The plant has a number of activities – antimicrobial, anthelmintic, antimalarial, hepatoprotective, antidiabetic, gastroprotective, anti-inflammatory, antioxidant, tumor-inhibitory etc. (**Street *et al.*, 2013**). The high protein content, carbohydrates, minerals, and vitamins make it a particularly valuable crop both for humans and animals (**Aldahak *et al.*, 2021**). The first attempts to grow *C. intybus* began 4000 years ago (**Wang and Cui, 2011**). The conventional method of vegetative propagation is limited by variations in abiotic and biotic environmental conditions. Micropropagation is a good alternative to provide quality stock plants, preserve valuable genotypes and forms, and overcome the genetic and phenotypic variability of wild-growing plants (**Debnath *et al.*, 2006**). In the literature, there are a number of studies concerning the micropropagation of *C. intybus*. Most authors are unanimous that organogenesis in chicory passes through the callus phase and the frequency of direct organogenesis is lower (**Abdin and Ilah, 2007**; **Dakshayini *et al.*, 2016**; **Doliński and Olek, 2013**). The commonly used cytokinins for shoot development *in vitro* of *C. intybus* are N^6-benzylaminopurine (BAP), kinetin, zeatin or thidiazuron (TDZ) usually in combination with some of the following auxins: α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) (**Doliński and Olek, 2013; Maroufi *et al.* 2012; Shahin *et al.* 2015; Dakshayini *et al.*, 2016**). Plants growing in wild populations vary in terms of morphology and content of biologically active substances (**Doliński and Olek, 2013**). *In vitro* micropropagation enables large-scale production of homogenous, disease-free plants producing phytochemicals with consistent yield and quality (**Espinosa-Leal *et al.*, 2018**). It is of interest to determine whether changes have occurred in the chemical composition of the micropropagated plants compared to the initial plants collected from the wild population. Often, the conditions of *in vitro* cultivation (high relative humidity, low ventilation rate, high concentrations of growth regulators, etc.) act as stress factors, causing changes in the secondary metabolism of plants. In the literature, knowledge of the chemical composition of micropropagated *C. intybus* plants is rather scarce. It was found that the combination and concentrations of plant growth regulators (PGRs) used in Gamborg's (B5) and Murashige and Skoog (MS) media were not only essential for the formation of callus, shoots, and roots but also caused a change in the amounts of some phenolic components (caftaric, chicoric, and chlorogenic acids and esculin) (**Abas *et al.*, 2023**). The high content of inulin and esculin from extracts of leaves and roots was detected in *in vitro* regenerated plants through HPLC (**Rehman *et al.*, 2003; Kumari *et al.*, 2007; Ohadi Rafsanjani *et al.*, 2011**). Most chicory phytochemical investigations are conducted using HPLC, but GC/MS is used when the emphasis is on non-polar compounds. Studies investigating the GC/MS-based metabolite profiles of *C. intybus* mainly refer to wild plants. Among the 78 different compounds identified in the methanol extract of wild chicory leaves, the main phytochemical constituents were phytol and stigmast-5-en-3-ol (**Malik *et al.*, 2017**). The 64 common metabolites were recognized from the leaves of 7 chicory specimens collected from different altitudes, with methyl commate B, gamma sitosterol, and 9, 12, 15-octadecatrienoic acid predominant (**Malik *et al.*, 2022**). The objective of this study was to develop an effective micropropagation protocol for *C. intybus*, and to compare the total phenolic content and metabolic profiles of micropropagated and wild-growing plants.

MATERIALS AND METHODS

Seed sterilization

The seeds were collected from plants growing in a wild population on the Vitosha Mountain, near the village of Bistritsa, Bulgaria. Seeds were washed with tap water and a detergent. Soaking seeds in 70% ethanol for 2 min followed by 0.1% mercuric chloride for 20 min was used for seed sterilization. Afterwards, three-fold rinses in autoclaved distilled water were performed. The sterilized seeds were cultured on **Murashige and Skoog, 1962** (MS) medium free of plant growth regulators (PGRs). Fifty seeds were used to test the germination *in vitro* in two replications.

Shoot multiplication

Axenic explants (stem segments excised from two-month-old *in vitro* germinated seedlings) were used for the initiation of *in vitro* culture. MS media supplemented with different types of cytokinins N^6-benzylaminopurine (BAP), kinetin (Kn), N_1-(2-chloro-4-pyridyl)-N_2-phenylurea (4PU-30), zeatin (Z) or N^6[2-isopentenyl]-adenine (2-iP) at concentration 1 mg/L, combined with the auxin α-naphthalene acetic acid (NAA) at concentration 0.1 mg/L were applied for induction of shoot multiplication. In addition, two nutrient media abbreviated $B_{0.5}N_{0.1}$ and $Kn_{0.5}N_{0.1}$,

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Article

Influence of the Abiotic Elicitors Ag Salts of Aspartic Acid Derivatives, Self-Organized in Nanofibers with Monomeric and Dimeric Molecular Structures, on the Antioxidant Activity and Stevioside Content in Micropropagated *Stevia rebaudiana* Bert.

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Abstract: The use of nanomaterials in biotechnology for the in vitro propagation of medical plants and the accumulation of certain biologically active metabolites is becoming an efficient strategy. This study aimed to evaluate the influence of the concentration (0, 1, 10, 50, and 100 mg L^{-1}) of two types of nanofibers on the growth characteristics, the antioxidant status, and the production of steviol glycosides in micropropagated *Stevia rebaudiana* Bert. plantlets. The nanofibers were synthesized by aspartic acid derivatives (L-Asp) Ag salts self-organized into nanofibers with two different molecular structures: monomeric, containing one residue of L-Asp with one hydrophilic head which bonds one Ag ion (NF1-Ag salt); and dimeric, containing two residues of L-Asp with two hydrophilic heads which bond two Ag ions (NF2-Ag salt). An increase in the shoots from the explants' number and length, biomass accumulation, and micropropagation rate was achieved in the plants treated with the NF1-Ag salt in concentrations from 1 to 50 mg L^{-1} after 30 days of in vitro proliferation compared to the NF2-Ag salt. In contrast, the plants grown on MS media supplemented with NF2-Ag salt exhibited an increase in the level of stevioside, rebaudioside A, and mono- (CQA) and dicaffeoylquinic (DCQA) acids as compared to the NF1-Ag salt.

Keywords: *Stevia rebaudiana* Bert.; antioxidant activity; in vitro propagation; steviol glycosides; monocaffeoylquinic acids (CQA); dicaffeoylquinic acids (DCQA)

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1. Introduction

Stevia rebaudiana Bertoni (sweet leaf, sweet herb of Paraguay, honey leaf, or candy leaf) is a native plant from South America and is one of the most preferred natural sweeteners in recent years. The plant's sweetness is due mainly to steviol glycosides, which are 250–300 times sweeter than table sugar. The stevioside and rebaudioside A (the two main diterpenoid steviol glycosides), are present in the plant's leaves. Since the body does not metabolize the glycosides in stevia, the leaves have been employed as a low-calorie sweetener [1]. The other compounds extracted from stevia leaves are flavonoids, alkaloids, chlorophylls, xanthophylls, hydroxycinnamic acids (caffeic, chlorogenic, etc.), oligosaccharides, free sugars, amino acids, and lipids [2,3]. The plant exhibits several biological activities including anti-hypertensive, anti-obesity, anti-diabetic, antioxidant,

Author Contributions: Conceptualization—M.G.; methodology, M.G., M.P., D.T. and A.T.; analysis—M.G., M.P., D.T., K.M.-G., M.S., T.N., E.K., V.I. and A.T.; data curation, M.G.; writing—original draft preparation, M.G.; writing—review and editing, M.G., K.M.-G., M.P., D.T. and A.T.; project administration, M.G. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data available in a publicly accessible repository.

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Review

Effects of Different Elicitors on Micropropagation, Biomass and Secondary Metabolite Production of *Stevia rebaudiana* Bertoni—A Review

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Abstract: *Stevia rebaudiana* Bertoni is a valuable plant whose products are increasingly used in medicine, pharmacy and the food industry. This necessitates the use of biotechnological approaches for its mass propagation. Establishing optimal conditions for *in vitro* cultivation is essential for obtaining high biomass and secondary metabolites production. A large number of articles considering the role of plant growth regulators and other additives in the culture medium in the growth and development of *Stevia* are available in the literature. However, there are no summarized data about the use of nanoparticles in *Stevia* tissue cultures. Therefore, this review also includes the research conducted so far on the effect of nanoparticles on *Stevia* micropropagation. Furthermore, the influence of different elicitors on secondary metabolite production and antioxidant activity of *in vitro*-cultivated *Stevia* plants have been discussed. By referring to the collected literature, we concluded that biotechnological approaches applied to *S. rebaudiana* cultivation might improve the agronomic traits of plants and steviol glycosides production.

Keywords: antioxidant activity; *in vitro*; nanoparticles; plant cell culture; plant growth regulators; stevioside

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1. *Stevia rebaudiana* (Bertoni)

1.1. Botanical Description

Stevia rebaudiana Bertoni is a herbaceous perennial plant of the Asteraceae family [1]. It is native to South America, in particular Brazil and Paraguay [2]. The plant is approximately 60–75 cm tall, the leaf is sessile and oppositely arranged, the flower is white and the seed is very small [3]. Dr Moises Santiago Bertoni first reported this plant in 1887 [3] when he learned of its unique properties from the Paraguayan, Indians and Mestizos [4].

1.2. Applications

Stevia is a natural sweetener plant known as Sweet leaf, Sweet herbs and Honey leaf [5–7]. The sweetness is due to the presence of more than 30 different steviol glycosides (SGs) found mainly in the leaves [2,8]. SGs are diterpenoids whose chemical structure is based on an aglycone core known as steviol (ent-13-hydroxyur-16-en-19-oic acid) to which a different number and types of sugar molecules are attached [9]. The four abundant SGs are stevioside, rebaudioside A, rebaudioside C and dulcoside A. The content of sweet components varies between 4 and 20% in the dry leaves [10]. Pure steviosides are non-caloric and 300 times sweeter than sucrose or cane sugar [3,11,12].

Stevia is used in many forms such as fresh and dried leaves, leaf powder, extracts and liquid concentrates [2]. The powdered form of the leaves has hypoglycemic and body-weight-reducing efficiency [13]. It has been recommended for diabetic and diet-conscious patients [3,13,14]. Stevia has been used in a wide range of processed foods as a substitute

The results obtained by the FRAP method led authors to assume that lysine in a higher concentration suppresses the effect of creatine—creatine increases the antioxidant activity measured by the FRAP method and lysine returns it closer to the control. The DPPH assay showed that creatine alone and in combination with lysine as a salt did not alter the overall antioxidant status of the plant.

10. Conclusions

Plant tissue culture techniques are increasingly used for the mass propagation of *S. rebaudiana* Bert. Cooperative applications of both traditional and biotechnological tools enable the generation of plants with desired agronomic traits such as disease resistance, improved biomass and high glycoside content. In recent years, many efforts have been made to clarify the role of nanoparticles as a novel elicitor for plants' primary and secondary metabolism. There are different and often conflicting reports on the effect of NPs on the growth and development of various plant species. Many questions concerning the fate and toxicity of NPs in plant cells remain unsolved.

Author Contributions: Conceptualization: K.M.-G. and M.G.; original draft preparation: K.M.-G. and M.G.; writing—review and editing, M.G., I.S., K.M.-G., M.P., E.K. and M.S.; project administration, M.G. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Not applicable.

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Abbreviations

2:4-D—2,4-dichlorophenoxyacetic acid; ALG—alginate; APX—ascorbate peroxidase; BAP—6-benzyl aminopurine; CAT—catalase; CH—casein hydrolysate; CHI—chitosan; CW—cococnut water; DPPH—2,2-diphenyl-1-picryl hydrazyl; DW—dry weight; FW—fresh weight; FRAP—ferric reducing/antioxidant power; GA3—gibberellic acid; GPX—guaiacol peroxidase; GR—glutathione reductase; IAA—indole-3-acetic acid; HPLC—high-performance liquid chromatography; iP—N6-(2-isopentenyl)-adenine; ISSR—inter simple sequence repeat; Kn—kinetin; LED—light-emitting diode; LS—Linsmaier and Skoog; ME—malt extract; Me-JA—methyl jasmonate; MS- Murashige and Skoog; NAA—α-naphthalene acetic acid; NPs—nanoparticles; PGRs—plant growth regulators; RAPD—Randomly amplified polymorphic DNA; ROS—reactive oxygen species; SA—salicylic acid; SGs-steviol glycosides; SOD—superoxide dismutase; TAC—total antioxidant capacity; TFC—total flavonoid content; TPC—total phenolic content; TRP—total reducing power; WPM—woody plant medium; YE—yeast extract; Z—zeatin.

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BIOLOGY

Plant physiology

IN VITRO MICROPROPAGATION OF *Helichrysum arenarium* (Asteraceae) AS A TOOL FOR INTRODUCING THE SPECIES IN AGRICULTURE

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Abstract

Helichrysum arenarium, known as sandy everlasting, is a perennial plant species, used in traditional medicine. In Bulgaria, it is protected by the Biodiversity Act and under a special regime of use based on the Medicinal Plants Act. Seeds from five Bulgarian populations were in vitro germinated and seedlings were sub-cultured on five media supplemented with different plant growth regulators (PGRs) and control MS medium free of PGR. The type of cytokinin turned out to be of crucial importance, as the presence of kinetin stimulated formation of rhizomes and direct organogenesis, BAP caused callogenesis, indirect organogenesis and hyperhydricity, and Meta-topolin – necrosis. Best results were obtained on medium with reduced macrosalt and sucrose concentrations as rhizomes gave rise to numerous well-developed and spontaneously rooted plantlets, up to 55 originating from a single seed. Plants were potted in soil mixture, adapted to phytotron conditions, and transferred to the greenhouse for acclimation, then planted on the ex situ collection. All plants flowered during the first summer outdoor. Nuclear DNA amount was measured by flow cytometry to check possible abnormalities of the in vitro multiplied plants. The estimated DNA amount varied in the range 1C = 0.85–0.89 pg. No deviations in the ploidy level were detected. No difference between the studied populations

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Effect of creatine and creatine lysinate *on the in vitro* cultivation and antioxidant potential of *Stevia rebaudiana* Bertoni and *Leontopodium alpinum* Cass

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Abstract
Stevia rebaudina Bertoni and Leontopodium alpinum Cass. (Asteraceae) are valuable plants, largely applied in traditional and formal medicine. Six variants of Murashige and Skoog media containing creatine or creatine lysinate at different concentrations (1, 5 and 10 mg/l) were used. The application of creatine lysinate in the MS nutrient media influenced positively the apical growth and elongation of stevia and edelweiss in vitro plants. The higher activities of the antioxidant enzymes (SOD, APX and GPX), as well as the higher content of flavonoids and phenols, were detected in stevia compared to edelweiss. The activity of CAT was much more pronounced in edelweiss.

The activities of CAT, APX and GPX were found to decrease with the addition of creatine or creatine lysinate to the culture medium while the activity of SOD increased as this tendency is better expressed in L. alpinum. The selected concentrations of creatine and creatine lysinate did not show statistically significant differences in either of the treatments compared to control in both the species measured by free radical scavenging capacity (DPPH method).

Keywords: Medicinal plants, micropropgation, antioxidant enzymes, amino acids.

Introduction

Interest in medicinal plants has increased over the past three decades because of their health benefits in terms of safety and cost compared to synthetic drugs[4,64]. *Stevia rebaudiana* Bertoni and *Leontopodium alpinum* Cass. are valuable medicinal plants belonging to Asteraceae family. *S. rebaudiana* is a perennial herb, native to the northern Republic of Paraguay and South America, typical for regions with tropical and subtropical climates[23]. It is a natural sweetener plant known as Sweet Leaf, Sweet Herbs and Honey Leaf[2,15].

The leaves of stevia are a source of diterpene glycosides such as steviosides and rebaudiosides, which are 200 to 300 times sweeter than sucrose or cane sugar[25]. Stevioside is known as a valuable natural sweetening agent attributed to its relatively good taste and chemical stability[47]. It had been suggested for diabetic patients because it is a non-calorie sweetener; the powdered form of stevia leaves has hypoglycemic and body weight reducing potencies[10,61].

Edelweiss (*Leontopodium alpinum* Cass.) is a traditionally used medicinal plant for the treatment of gastrointestinal disorders such as diarrhea, dysentery and colic as well as bronchitis, angina and fever[19]. Various compounds such as terpenoids, phenylpropanoids (phenolic acids, flavonoids, coumarins, lignans), fatty acids and polyacetylenes isolated from different parts of edelweiss determine the pharmacological effects of the plant [20,24,56]. The species is native to the Pyrenees, the Alps, the Carpathians and the Balkan Peninsula and is enclosed within the Red Book of Bulgaria.

The high pharmacological potential of both species necessitates their cultivation. Conventional methods of stevia vegetative propagation are limited to a low seed germination percentage because of self-incompatibility and the low number of individuals that may be obtained from a single plant and successfully adapted to the soil[50,55]. Micropropagation, or *in vitro* culture appears to be the best method to overcome those problems and has the potential to provide giant numbers of plants in a brief time [50,53]. There are several studies of the micropropagation of *S. rebaudiana*[3,5,33,38,44,46,51]. Stevia can form multiple shoots from the nodal explants and appears to be appropriate for large-scale production[53]. In the literature, there are some reports on the *in vitro* cultivation of *L. alpinum* through direct or indirect regeneration using apical buds from mature, senescing plants or *in vitro* obtained seedlings[31,45].

The growth and morphogenesis of plant tissues under *in vitro* conditions are largely influenced by the composition of the culture media[35]. In the plant nutrient medium, nitrate ions, ammonium salt, amino acids and complex organic compounds supply nitrogen[8]. Amino acids provide plant cells with a source of nitrogen that is easily assimilated by tissues and cells faster than inorganic nitrogen source. They affect many physiological processes in plants by participating in the regulation of metabolic pathways, acting as intermediates of the final metabolites in certain metabolic pathways[71]. Amino acids used for enhancement of cell growth in culture media included glycine, glutamine, asparagine, L-arginine, cysteine and L-tyrosine[65]. Amino acids have been used as an organic nitrogen source in *in vitro* cultures of many species such as alfalfa, maize, sorghum, pineapple, rice, *Artemisia vulgaris* etc. to increase somatic embryogenesis and regeneration potential[16, 27, 28, 36, 52, 60].

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Review article

Employment of nanoparticles for improvement of plant growth and development

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Abstract

Kirova E., Geneva M., Petrova M., Miladinova-Georgieva K., Sichanova M., 2022: Employment of nanoparticles for improvement of plant growth and development. – Botanica, 28(2): 113–132. https://doi.org/10.35513/Botlit.2022.2.4

Nanotechnology, with its research and results, has become one of the essential fields among scientific disciplines. Nanotechnology is used in many areas of science such as physics, chemistry, pharmacology, materials science, medicine and agriculture. The use of nanotechnology could provide breakthroughs that would revolutionise many scientific studies. The role of nanoparticles in plant nutrition under soil pollution represents a comprehensive overview of nanotechnology in agriculture related to the importance, recycling, and transformation of nanoparticles. Nanomaterials are used in plant protection, nutrition, and the management of agricultural practices. The new challenges nanotechnology faces today include using biological or green synthesis methods to produce nanoparticles and offset the toxicity of conventionally integrated nanoparticles. The efficiency of nanoparticle uptake and the effects of nanoparticles on growth and metabolic functions differ between plant species. The concentration of nanoparticles affects processes such as germination and plant growth and development. The agriculture sector has also profited from various nanotechnology-based products such as nanofertilisers, nanopesticides, nanogrowth promoters, and many more for sustainable agriculture and crop improvement.

Keywords: crops, nanocomposites, nanomaterials, nanopesticides, yield.

Abbreviations: Nanoparticles (NPs), Silver nanoparticles (AgNPs), Zinc nanoparticles (ZnNPs), Quantum dots (QDs), Titanium dioxide nanoparticles (TiO_2), Carbon nanotubes (CNTs), Silicon nanoparticles (SiNPs), biochar-based nanocomposites (BNCs), Nanomaterials (NMs)

INTRODUCTION

The use of nanotechnology is increasing in almost every dimension of the plant, animal worlds, and among humans. The global market for nanomaterials may reach up to USD 100 billion by 2025, indicating a wider use of nanotechnology in every application area (Allan et al., 2021). Nanoparticles are increasingly used to enhance crop productivity, abiotic and biotic stress tolerance in plants, nanofertilizers, biosensors, cancer therapy, nanomedicines, cosmetics, electronics, and waste treatment. Nanoparticles affect plants at morphological, anatomical, biochemical, and molecular levels. (Siddiqui et al., 2022).

Agriculture is the most fundamental and stable sector supporting industrial growth and the economy, as it produces raw materials for the food and feed industries. Limitations in natural resources (produc-

FULL PAPER

Developmental and Environmental Effects on Sesquiterpene Lactones in Cultivated *Arnica montana* L.

by **Milka Todorova**[a]), **Antoaneta Trendafilova***[a]), **Antonina Vitkova**[b]), **Maria Petrova**[c]), **Ely Zayova**[c]), and **Daniela Antonova**[a])

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The amount of sesquiterpene lactones and the lactone profile of *Arnica montana* L. in flowering and seed formation stages *in vitro* and *in vivo* propagated from seeds of German, Ukrainian, and Austrian origin and grown in two experimental fields were studied. It was found that *in vitro* propagated 2-year plants in full flowering stage accumulated higher amount of lactones in comparison to *in vivo* propagated 3-year plants and to the seed formation stage, respectively. Helenalins predominated in *in vivo* propagated 2-year or *in vitro* propagated 3-year plants. 2-Methylbutyrate (2MeBu) was the principal ester in the samples with prevalence of helenalins, while isobutyrate (iBu) was the major one in the samples with predominance of 11,13-dihydrohelenalins. The results revealed that the environmental conditions on Vitosha Mt. are more suitable for cultivation of *A. montana* giving higher content of lactones.

Keywords: *Arnica montana*, Asteraceae, Sesquiterpene lactones, Helenalins, 11,13-Dihydrohelenalins, Developmental and environmental factors.

Introduction

Arnica montana L. is an endemic species to Europe where it is found from Norway to the Balkans and from Spain to Ukraine [1]. In some countries, the populations of the species are still stable, while in others, the areas of distribution decreased because of large consumption or other human intervention in natural habitats [2], and hence, it is included in the lists of threatened and endangered species [3]. The existence of this species in Bulgaria in Rila Mountain was described earlier but the location was not specified. Unfortunately, its survival through the years is not confirmed [4]. To avoid the risk of extinction, as well as to ensure standardized raw material for pharmaceutical industry, many experiments for cultivation have been carried out [5 – 9].

Arnica montana is applied to people with arthritis, rheumatic disorders, inflammation bruises, sprains, soreness, and swelling/muscle spasms from sports activity; arthritis set off by seasonal change; and general muscle and joint pain [10][11]. It is used as tincture, cream, or ointment for external applications mainly [12] and as homeopathic pills [13]. Phytochemical research revealed that this species is rich in sesquiterpene lactones of helenalin and 11,13-dihydrohelenalin types which are responsible for the biological activity [14][15]. It has been shown that sesquiterpene lactones are responsible for anti-inflammatory activity [16] and inhibition of transcription factors NF-κB and NF-AT [11][14][17]. Minimum content of 0.4% sesquiterpene lactones is required by the European Pharmacopoeia [18] in *Arnica flos* as a medicinal plant. In addition, antioxidant and antimicrobial activities of the herb are attributed to flavonoids and phenolic acids [19].

Attempts for cultivation of *A. montana* are directed toward optimizing the conditions for improving the quality of the herb, *i.e.*, increasing the content of sesquiterpene lactones, especially the content of helenalins, which are reported as the most active lactones in the species [11][15]. This work aims to assess the influence of different developmental and environmental factors on the accumulation of these secondary metabolites, the lactone profile, and helenalins/11,13-dihydrohelenalins ratio (H/DH) in *A. montana*. To achieve the goal of this study, the following factors were investigated: plant age (two and three years), stage of development (full flowering and seed formation), origin of seeds for propagation (Germany, Ukraine, and Austria), method of propagation (*in vitro* and *in vivo*), and environmental conditions (experimental fields – Beglika on Rhodope Mts. and Zlatni mostove on Vitosha Mt.).

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Effect of Medium Salt Strength on the Micropropagation, Phenolic Content and Antioxidant Activity of *Arnica montana* L., Threatened Plant Species

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ABSTRACT: The effect of different strength of Murashige and Skoog (MS) medium (full, half-, third and quarter content of salts and vitamins) on micropropagation, shoot growth, in vitro rooting and antioxidant properties of *Arnica montana* was demonstrated. The full strength MS medium supplemented with 1 mg L^{-1} 6-benzylaminopurine (BAP) and 0.1 mg L^{-1} indole-3-acetic acid (IAA) was the best for plant multiplication (11.4 number of shoots per explant) and biomass production (FW 1.66 g and DW 0.25 g). Optimal rooting medium was half strength MS rooting medium contained 0.5 mg L^{-1} indole-3-butyric acid (IBA), although the maximum number of roots was induced on quarter strength MS rooting medium. The rooted plants were successfully ex vitro acclimatized. The highest phenolic content and antioxidant activity were recorded in shoots grown on ¼ strength of MS medium, whether derived from micropropagation or from rooting stage. This study showed that tissue culture of *A. montana* is alternative source of natural antioxidants.

Keywords: *A. montana*; in vitro culture; shoot growth; phenols; antioxidants.

INTRODUCTION

Arnica montana L. is a valuable medicinal plant spread in various regions of Europe (Lange, 1998). It is a rare and endangered species according to International Union for Conservation of Nature (Bilz *et al*., 2011). The plant is rich in sesquiterpene lactones, flavonoids, phenolic acid, essential oils etc. (Willuhn, 1998; Merfort, 2007). *A. montana* has a long history of use in the folk medicine, and presently is largely applied in pharmacy and cosmetics due to its antiseptic, anti-inflammatory, antibacterial, antifungal and antioxidant activities (Shiffman *et al.*, 2012). Development of biotechnological methods is of a great importance for multiplication and conservation of this endangered species and for enhancement of biologically active compounds particularly those with antioxidant properties. For micropropagation, most widely used culture medium is Murashige and Skoog, 1962 (MS) medium, because most of the plants respond favorably

to this medium, contained all the nutrients essential for in vitro plant growth (Kumar and Reddy, 2011). Full strength of salts in media provide good results for numerous species, but in some plants and for specific purposes the reduction of salts level to half or quarter of the full concentration gave better results in in vitro growth (Saad and Elshahed, 2012). Most of the authors studied in vitro cultures of *A. montana* recommended using half strength MS medium for rhizogenesis (Conchou *et al.*, 1992; Le, 2000; Petrova *et al*., 2011), however the effect of strength of the MS medium on micropropagation and rooting of the species is not sufficiently

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Influence of carbon sources on growth and GC-MS based metabolite profiling of *Arnica montana* L. hairy roots

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Abstract: *Arnica montana* L. (Asteraceae) is an economically important herb that contains numerous valuable biologically active compounds accumulated in various parts of the plant. The effects of carbon sources (sucrose, maltose, and glucose) at different concentrations (1%, 3%, 5%, 7%, and 9%) on growth were studied and GC-MS based metabolite profiling of *A. montana* hairy roots was conducted. The optimal growth and biomass accumulation of transformed roots were observed on an MS nutrient medium containing 3% or 5% sucrose. GC-MS analysis of hairy roots of *A. montana* showed the presence of 48 compounds in polar fractions and 22 compounds in apolar fractions belonging to different classes of metabolites: flavones, phenolic acids, organic acids, fatty acids, amino acids, sugars, sugar alcohols, hydrocarbons etc. Among the various metabolites identified, only the sugars and sugar alcohols were influenced by the concentration of the respective carbon sources in the nutrient medium.

Key words: *Arnica*, transformed roots, carbon sources, GC-MS analysis

1. Introduction

The perennial herb *Arnica montana* L. (Asteraceae) is one of the most important medicinal species worldwide. The plant is used for treatment of hematomas, contusions, sprains, rheumatic disease, and superficial inflammations of the skin (Willuhn, 1998). Its pharmacological value is due to the presence of sesquiterpene lactones, flavonoids, essential oils, and other active compounds in various parts of the plant. The roots contain essential oils, phenolic acids, oligosaccharides, lignans, etc (Willuhn, 1972a, 1972b; Rossetti et al., 1984; Schmidt et al., 2006; Pljevljaušić et al., 2012). The chemical composition of hairy roots of *A. montana* obtained by genetic transformation of plant tissue with *Agrobacterium rhizogenes* is less studied. There are few publications related to transformed roots essential oil constituents (Weremczuk-Jezyna et al., 2006, 2011). The greatest advantages of hairy roots are their genetic and biochemical stability, fast auxin-independent growth, and the ability to synthesize natural compounds at levels comparable to intact plants. These are the reasons that in recent years hairy roots of various medicinal and aromatic plants are being cultured for the production of secondary compounds (Murthy et al., 2008; Danphitsanuparn et al., 2012; Nagella et al., 2013; Thiruvengadam et al., 2014; Shakeran et al., 2015). It is known that the addition of a carbon source in the nutrient medium is necessary for the growth of tissue cultures, including hairy roots, because plants autotrophic ability at in vitro conditions is limited. It was recently found that sugars (monosaccharide and disaccharide) induce signals that affect metabolism, development, growth, and gene expression of plants (Praveen and Murthy, 2012). The production of numerous biologically active compounds through hairy roots (such as hyoscyamine, isoflavones, sennosides A and B, pyranocoumarins, gymnemic acid, ginsenoside) was found to be affected by initial concentrations of carbon sources in the nutrient media (Liang et al., 2004; He et al., 2005; Pavlov et al., 2009; Xu et al., 2009; Romero et al., 2009; Nagella et al., 2013; Kochan et al., 2014). To date, there have been no studies regarding the effects of type and concentration of sugars on growth and accumulation of metabolites of hairy root cultures of *A. montana* and this motivated our study. By manipulating the type and concentration of the carbon sources, our goal was to determine the optimal conditions for promoting growth and biomass accumulation of hairy roots and improving the synthesis of important secondary metabolites in them.

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ENHANCEMENT OF *ARNICA MONTANA IN-VITRO* SHOOT MULTIPLICATION AND SESQUITERPENE LACTONES PRODUCTION USING TEMPORARY IMMERSION SYSTEM

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Keywords:

Agar-gelled medium, Liquid medium, TIS.

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ABSTRACT: *Arnica montana* L. (Asteraceae) is a valuable medicinal plant species, endemic to Europe, which is threatened in many countries due to its overharvesting. The increasing market demand requires development of an effective method for *A. montana* rapid propagation, offering the possibility for its field cultivation as an alternative to plant gathering from nature. Three *in -vitro* culture systems were compared to determine the best shoot multiplication of *A. montana*: agar-gelled medium, static liquid medium and Temporary Immersion System (TIS RITA®). Murashige and Skoog (MS) nutrient medium supplemented with 1 mg/l BA and 0.1 mg/l IAA was used in all experiments. The highest micropropagation rate (18.2 shoots/explant, for 5 weeks) was obtained via TIS culture. Besides, these plants showed higher sesqiuterpene lactones content than those derived from the other tested systems. The shoots were successfully *in -vitro* rooted and *ex-vitro* adapted. The elaborated method for mass multiplication of *A. montana* allows significant enhancement of the *in -vitro* process and could be applied to produce planting material, thus contributing to the conservation of the species as well.

INTRODUCTION:

Arnica montana L. (Asteraceae) is a medicinal plant, largely applied in pharmacy and cosmetics. The species is rich in sesquiterpene lactones and possess antiseptic, anti-inflammatory, antibacterial and antioxidant effects[1]. *A. montana* is endemic to Europe, included in the European Red List of Vascular Plants (LC), and threatened in many European countries due to loss of habitats and overharvesting from natural populations[2].

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The plant deficiency stresses the need of elaboration of effective propagation methods. Several protocols have been reported on *in-vitro* multiplication of *A. montana* on agar gelled medium differing by their efficiency; however, the propagation rate needs to be improved [3-5]. The conventional *in-vitro* propagation method on agar medium requires a considerable amount of handwork: shoots separation and regular sub-culturing on fresh medium every 4 to 6 weeks[6].

The recent trend is changing the state of the nutrient medium to liquid which allows automation and commercialization of the *in-vitro* process. Applied tissue culture technique for micropropagation of several plant species in liquid medium was found to be more appropriate for mass

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Comparative study of *in vitro, ex vitro* and *in vivo* grown plants of *Arnica montana* – polyphenols and free radical scavenging activity

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Abstract – *Arnica montana* L. is an endangered species rich in sesquiterpene lactones, phenolic acids and flavonoids with high pharmaceutical value. The polyphenolic content and free radical scavenging activity of plants that had passed all stages of cultivation: micropropagation and rooting (in vitro), adaptation in greenhouse (ex vitro) and mountain conditions (in vivo) were evaluated. Four surface flavonoid aglycones [scutellarein 6-methyl ether (hispidulin), scutellarein 6,4'-dimethyl ether (pectolinarigenin), 6-OH luteolin 6-methyl ether and kempferol-6-methyl ether] were detected in the acetone exudates of the studied samples by means of thin layer chromatography. No differences in the accumulation of surface flavonoids were found among the tested leaf extracts of in vitro, ex vitro and in vivo samples. However, the extracts from the flowers were richer in surface flavonoids than extracts from the leaves. The methanol extracts of the samples from ex vitro and in vivo grown *A. montana* plants had significantly higher radical scavenging activity and polyphenolic content than the extracts of in vitro samples. The observed differences in the contents of these biologically active compounds were related to different growth conditions and stages of plant development. The biotechnological method of *A. montana* established holds promise for the future production of antioxidants.

Keywords: antioxidant, flavonoid, phenol, *Arnica montana*

Introduction

Arnica montana L. (Asteraceae) is a valuable perennial herb. The species contains sesquiterpene lactones (e.g. helenalin), phenolic acids (caffeic acid derivatives) and flavonoids (quercetin 3-*O*-glucuronic acid) with significant antiseptic, anti-inflammatory, antibacterial and antioxidant effects (WOERDENBAG et al. 1994, LYSS et al. 1997, IAUK et al.

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REVIEW

Biotechnological approaches for cultivation and enhancement of secondary metabolites in *Arnica montana* L.

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Abstract *Arnica montana* L. (Asteraceae) is a valuable medicinal plant with anti-inflammatory and cicatrizing properties attributed to the sesquiterpene lactones, flavonoids and essential oil produced in the flower heads. In many European countries, the populations of *A. montana* are close to extinction in their natural habitats because of uncontrolled eradication and indiscriminate collection of the plants. Various approaches for in vitro propagation of the species and also, biosynthesis of secondary metabolites in tissue and cell cultures are assessed in the current review. Special attention is paid to the biological activity and chemical composition of compounds produced by the species as well as the opportunities of in vitro methods to isolate high-productive clones. The influence of different factors on the micropropagation, callusogenesis, genetic transformation and identification of certain biologically active substances is discussed in detail. By the reference to the available issues we concluded that biotechnology applied to *A. montana* cultivation may improved the plant preservation and increased the production of sesquiterpene lactones and other secondary metabolites.

Keywords Micropropagation · Plant growth regulators · Hairy roots · Sesquiterpene lactones

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Introduction

Arnica montana L. (Asteraceae), an old established herbal plant has been used over the years in traditional medicine in Europe and North America (Willuhn 1991; Nichterlein 1995). This endangered species grows in northern and central European mountainous grasslands (Delabays and Mange 1991). Genus Arnica comprises about 32 species divided into five subgenera—*Andropurpurea*, *Arctica*, *Austromontana*, *Chamissonis* and *Montana* (Maguire 1943). *Arnica montana* flower heads have valuable properties due to the presence of sesquiterpene lactones of helenalin and 11α, 13-dihydrohelenalin type—the major ingredients in anti-inflammatory preparations. For these reasons, *A. montana* has been excessively collected and became rare. Increased demand for plant material is resulting in the eradication of its natural plant populations and therefore, the species is considering vulnerable or threatened with extinction. For commercial and ethical reasons it is important to protect and renovate *A. montana* populations assuring supply of plant material of sustainable sources. In many European countries, *A. montana* cutting, collecting, picking, uprooting, trading, either as fresh or as dried material is strictly forbidden (Lange 1998). The plant grows in nutrient-poor siliaceous meadows up to nearly 3,000 m altitude. In Bulgaria, the species was found to be distributed locally in Rila Mountain (Assyov et al. 2006). However, this information is not confirmed to date, despite the proper climate conditions in more upland regions. After attempt to breed *A. montana*, the first cultivar "ARBO" was developed (Bomme 1993) and today, it is commercially available. *A montana* cultivation is difficult and nonprofitable (Delabays and Mange 1991; Bomme et al. 1995) while established populations can be strongly adapted to the local environment; therefore, the

Application of modern analytical methods (HPLC, GC/MS and ^{1}HNMR) showed that this valuable medicinal plant is extremely rich in sesquiterpene lactones of helenealin and dihidrohelenalin type, essential oils, flavonoids, phenolic acids etc. However, the secondary metabolites production of *A. montana* grown in different mountain areas depends on the climate, altitude and development stages, which could result in change of the quantity and quality of the secondary products. The medicinal and commercial benefits of *A. montana* and its endangered status highlight the need of conservation and propagation of the plant. This review gathers different biotechnological approaches for in vitro cultivation of *A. montana* as ways for producing secondary metabolites. At present several protocols for *A. montana* micropropagation show it is possible to overcome some of the inherent problems of aseptic culture establishment and induction of multiple shoots. One way to investigate the in vitro bioactive product accumulation is to develop a culture of transformed organs such as hairy roots capable of producing bioactive substances. A more efficient transformation protocol needs to be established for *A. montana* because transformation frequency is still low. A detailed chemical profile of secondary metabolites from in vitro cultures, especially hairy roots, is required as well. The callus and suspension cultures also could be used for secondary metabolites extraction. However, the new challenges the plant biotechnology industry faces today include cost efficiency, control and optimization of growth conditions, etc. A recent trend is changing the state of the nutrient medium from agar solidified to liquid, which allows automation and commercialization of the in vitro processes and the researchers should focus on these studies. The production of secondary metabolites could be enhanced using bioreactors for large scale synthesis of the biologically active substances of *A. montana* for pharmaceutical and cosmetic industries.

Author contribution Mariya Petrova and Ely Zayova gathered the literature, wrote the manuscript and bear the basic responsibility for the final content. Roumiana Vassilevska-Ivanova and Mariana Vlahova focused on the reference, spelling and formal style of the article. All authors have read and approved the final manuscript.

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IN VITRO PROPAGATION OF *ARNICA MONTANA* L.

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Abstract

Arnica montana L. is a perennial herbaceous plant from the family of Asteraceae. It is widely spread in North Europe, the Alps and other mountains between 1000 m and 2500 m altitude. The species is included in the List of protected plants in many countries. In Bulgaria *A. montana* is not spread as a wild species and is very rarely cultivated. The plant is used as antiinflammation drug stimulating wound healing. It also possesses antirheumatic, antiseptic and antitumour effects.

The purpose of this study was to examine various explants and nutrient media in order to elaborate an efficient method for *A. montana* in vitro micropropagation. Over ten nutrient media were tested; the most appropriate among them for seed germination and explant regeneration being identified. The nutrient medium of Murashige and Skoog, 1962 supplemented with 1 mg l^{-1} benzyl adenine and 0.1 mg l^{-1} indolyl acetic acid was most suitable for micropropagation. The mean number of shoots per explant reached 7.46 and some explants gave rise to 16 shoots.

Key words: arnica, micropropagation, medicinal plant

Abbreviations: BA – N^6-benzyl adenine, 2-ip – N^6-(Δ^2-isopentyl) adenine, GA_3 – gibberellic acid, IAA – indolyl-3-acetic acid, NAA – α-naphthyl acetic acid, MS – Murashige and Skoog medium.

Introduction. *Arnica montana* L. is a perennial herbaceous plant from the family of Asteraceae. It is widely spread in North Europe, the Alps and other mountains between 1000 m and 2500 m. This species is included in the List of protected plants in Germany, France, Romania, Russia and other countries. In Bulgaria *A. montana* is not spread as a wild species and is quite rarely cultivated. In the remote past *A. montana* was traditionally used in medicine in Europe and North America. Usually the flowers are applied in medical practice while the leaves and the roots are rarely used. The plants contain certain very important biologically active substances: sesquiterpene lactones of the helanin type, flavonoids and the bitter non-glicoside compound arnicin, essential oils, tannins, etc. The plant is used as antiinflammation drug stimulating