BULGARIAN ACADAMY OF SCIENCES

INSTITUTE OF MICROBIOLOGY

„STEPHAN ANGELOFF“

SUMMERY

On a dissertation for the award of educational and scientific degree "Doctor"

in the field of 5.11. Biotechnology (Technology of biologically active molecules)

GERGANA SPASOVA KRASTEVA

тема

Plant bioengineering systems from *Gardenia jasminoides* Ellis for the production of active ingredients

SCIENTIFIC SUPERVISOR: Dr. Vasil Georgiev

SCIENTIFIC JURY:

Prof. Dr. Lyudmila Vladimirova Kabaivanova

Assoc. Prof. Dr. Galina Dinkova Stoyancheva

Prof. Dr. Kaloyan Kirilov Petrov

Assoc. Prof. Dr. Dasha Spasova Mihaylova

Assoc. Prof. Dr. Radka Zapryanova Vrancheva

Plovdiv , 2025

The dissertation is written in 176 pages. It is illustrated with 35 figures and 32 tables. 257 literary sources are cited. The dissertation was approved and scheduled for defense by a national scientific seminar on "Applied Microbiology and Microbial Biotechnologies" at the Institute of Microbiology "Stefan Angelov" - BAS, whose meeting took place on .....

The abstract is written in 80 pages and is illustrated with 29 figures and 26 tables. The numbering of the figures and tables does not correspond to that in the dissertation.

The PhD student was authorised with the right to defend the doctoral thesis by Order No. I-122/28.09.23, of the Director of the Institute of Microbiology-BAS, Prof. Dr. Penka Petrova

The defense of the dissertation will take place at an open session before a scientific jury on . ..... 2025, from ..... hours in the hall of the Institute of Microbiology "Stefan Angelov", BAS, ul. "Akad. G. Bonchev", bl. 26, Sofia.

The materials for the defense are available in the office of the scientific secretary of the Institute of Microbiology "Stefan Angelov", BAS, ul. "Akad. G. Bonchev", bl. 26, Sofia, and are published in accordance with the ZRASRB at www.microbio.bas.bg.

DNA - Deoxyribonucleic acid

NAA- 1-naphthylacetic acid

BAP - 6-benzylaminopurine

HPLC - High-performance liquid chromatography

GAE - gallic acid equivalent

DB - dry biomass

ABS - accumulated dry biomass

IBS - dry biomass growth index

B5 - 3-[(2,4-dihydroxy-3,3-dimethylbutanoyl)amino]propanoic acid

Na - number of alleles

Ne - effective number of alleles

He - expected heterozygosity

PIC - polymorphic information content

Ho - observed heterozygosity

DPPH - 2,2-diphenyl-1-picrylhydrazyl- 2,2-diphenyl-picrylhydrazyl

CUPRAC - Cupric reducing antioxidant capacity - Copper reducing antioxidant activity

FRAP - Ferric ion reducing antioxidant power - Iron reducing antioxidant activity

TEAC - Trolox equivalent antioxidant capacity - Trolox equivalent antioxidant activity

SSRs - Simple sequence repeats - Markers analyzing simple repeats of the DNA sequence

WP – Woody plant medium

MS - Murashige and Skoog medium

Content

INTRODUCTION

Plant cell cultures are considered the most promising technology for sustainable production of plant biomass. Extraction of valuable metabolites with the lowest environmental impact is a priority of modern science. In recent years, many plant species have been cultivated *in vitro* for commercial purposes. Most often for the needs of the pharmacy, but they are increasingly used in the food and cosmetic industries.

Gardenia jasminoides Ellis is a plant species with great economic value - not only in medicine and the food industry, but also as an ornamental plant. That is why there are a number of studies studying the possibilities of micro-propagation of the plant species. The plant is rich in iridoid glycosides, flavonoids, saffron glycosides, phenolic acids. It is important to note that there is a lack of data in the literature for a detailed study of the chemical composition of the different parts of the plant.

When cultivating plant *in vitro* cultures with the aim of extracting valuable ingredients, it is important to be familiar with their genetic stability. The occurrence of somoclonal variability during in vitro cultivation of the plant has attracted great interest in recent years. It is believed that this has a direct impact on the production of secondary metabolites. There are no detailed studies on the influence of the composition of the nutrient medium, the duration of cultivation, light and the growth regulators used, and their influence on the genetic stability of in vitro cultures. To date, there is no published information in the world database on the causes that induce somoclonal variability and the factors that influence it, in differentiated and dedifferentiated *in vitro* cultures of jasmine gardenia.

I. PURPOSE AND OBJECTIVES OF THE DISSERTATION

The present dissertation aims to study the effects of the duration of cultivation and the content of basic nutritional components, on the biosynthesis of phenolic compounds and somoclonal variability of differentiated and dedifferentiated in vitro systems from the jasmine gardenia plant (*Gardenia jasminoides* Ellis).

OBJECTIVES OF THE DISSERTATION

1. Obtaining differentiated and dedifferentiated *in vitro* cultures

2. Comparing the phytochemical profile of the jasmine gardenia plant with the *in vitro* systems commissioned by it

3. Tracking the dynamics of development of cell suspension culture and seedling culture of jasmine gardenia

4. Optimization of the growth conditions and development of cell suspension culture and seedling culture of jasmine gardenia

5. Tracking the genetic stability of in vitro systems, obtained from jasmine gardenia. Analyzing the causes of changes that occur during prolonged cultivation and when the concentration of basic components of the culture medium changes.

II. MATERIALS AND METHODS

1. Plant material

For the purposes of this work, a developed Gardenia jasminoides Ellis plant of certified origin was used, purchased from Hot Plant Company, Jackdaws Field Nursery, West Sussex, UK (https://www.hotplantco.co.uk).

1.1 Preparation of seedling culture

To obtain the in vitro systems, nodal segments containing one leaf node with two leaves were used as explants. The explants were sterilized by surface treatment with 70% ethanol for 30 seconds, followed by intrinsic sterilization in 4% calcium hypochloride for 30 minutes. The sterile explants were washed three times with sterile distilled water, dried on sterile filter paper and plated on sterile in vitro plant initiation medium. The medium is agarized (with 5% agar-agar) MS medium supplemented with 30 g/L sucrose and 4.0 mg/L 6-benzylaminopurine (BAP). It is cultivated at 26°C and photoperiod of 16 hours light and 8 hours dark until the formation of healthy in vitro plants.

1.2 Preparation of callus culture

To obtain callus culture, developed leaves were used as explants. The explants were sterilized by surface treatment with 70% ethanol for 10 seconds, followed by intrinsic sterilization in 4% calcium hypochloride for 15 minutes. The sterile explants were washed three times with sterile distilled water, dried on sterile filter paper, cut into 1 cm segments and plated on sterile callus initiation medium. The medium is agarized (with 5% agar-agar) 1/2MS medium supplemented with 30 g/L sucrose, 2.0 mg/L 1-naphthylacetic acid (NAA) and 0.5 mg/L 6-benzylaminopurine (BAP). It is cultivated at 26°C, in the dark, until a homogeneous callus mass is formed. The resulting callus is separated for independent development and maintained under the same conditions with a subculturing regime of 30 days.

1.3 Preparation of cell suspension

To obtain a cell suspension, loose sections of the callus culture are plated on sterile liquid medium for initiating cell suspension. The medium is liquid 1/2MS medium supplemented with 30 g/L sucrose, 2.0 mg/L 1-naphthylacetic acid (NAA) and 0.5 mg/L 6-benzylaminopurine (BAP). It is cultivated in 2-liter Erlenmeyer flasks on a rotary shaker at 115 rpm, at 26°C, in the dark, until a homogeneous cell suspension is formed. The resulting suspension is maintained under the same conditions with a subculture regime of 7 days.

2. Isolation and analysis methods

2.1 Analysis of primary and secondary metabolites of in vitro cultures of gardenia

2.1.1 Gas chromatography

Gas chromatography for identification of the metabolic profile of Gardenia jasminoides Ellis leaves and in vitro cultures at different degrees of differentiation

The extraction of biomass for gas analysis was performed according to the methodology described by Nikolova et al. (Nikolova et al. 2019). The obtained extractions and fractions were silicified according to Berkov et al. (Berkov, 2018).

2.1.2 HPLC analysis for identification of secondary metabolites

Approximately 500 mg of sample (callus suspension, sprout culture or callus) were used for three times methanol extraction (3 x 10ml 70% methanol) in an ultrasonic bath for 30 min. After filtration, the methanol was evaporated (45°C, under vacuum) and the aqueous fraction was brought to a volume of 50 ml with distilled water. Of the thus obtained extract, 20 ml was used for concentration of the sample by Strata C18-E (55um, 70A, 500mg/l mL) SPE (Phenomenex Inc., USA) following the manufacturer's procedure. The polar fraction was removed with 20ml distilled water and the phenolic fraction was eluted with 2ml methanol. The thus obtained fraction was used for HPLC analysis and antioxidant activity analysis.

The obtained extracts were analyzed by high-performance liquid chromatography as described by Mladenova et al. (Mladenova et. al.,2021).

3. Antioxidant activity

The antioxidant activity of the methanol extracts was analyzed using Trolox equivalent antioxidant activity (TEAC), 1.1-diphenyl-picrylhydrazyl (DPPH), Iron-reducing antioxidant activity (FRAP), Copper-reducing antioxidant activity (CUPRAC), Method for determining total polyphenol content (Folin-Ciocalteu) (Georgiev, V. et al., 2019) with modifications described in detail by Krasteva and colleagues (Krasteva, G., 2022).

4. Determination of growth and development dynamics

4.1 Growth and development dynamics of cell suspension cultures

A fresh seed of a cell suspension culture of gardenia was used to conduct the experiment. 2 liter Erlenmeyer flasks containing fresh inoculum were used on a rotary shaker at 115 rpm, at 26°C, in the dark, for 21 days. MS medium (Murashiga and skoog) was used with growth regulators 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine, 30g/L sucrose. Fresh biomass was then collected, dried and extracted for determination of accumulated dry biomass, HPLC analysis, antioxidant activity analysis and determination of somaclonal variability.

4.2 Growth and development dynamics of seedling cultures

For the experiment, RITA type temporary stirring bioreactors are used, containing 200ml liquid nutrient medium (Murashiga and skoog) with growth regulators 2.0mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine, 30g/L sucrose. 10g. fresh inoculum is used. The experiment lasts 35 days.

4.3 Determination of accumulated dry biomass (ADB)

The dry matter content of the biomass was determined by a direct method, whereby approximately 1g. (T1) of the collected fresh biomass was dried at 50 ± 2°C to obtain a constant weight (T2). The samples were tempered in a desiccator after drying and weighed. Then the results are calculated according to the equation

Weight (g) = [(T1 – T2)/T1]

Weight (%) = [(T1 – T2)/T1] * 100

4.4 Dry biomass growth index (DBGI)

DBGI = DGB/dry biomass

4.5 Nitrate, ammonium and phosphate ion uptake

Nitrate, ammonium and phosphate ions were determined by chemical test combinations (MERCK, Germany, cat. no. 1.14773.0001, 1.14752.0001, 1.14842.0001). The uptake is expressed as the degree of uptake, using the equation: Degree of uptake = (Final concentration / Initial concentration) × 100.

5. Optimization of the aeration regime for the cultivation of jasmine gardenia seedlings

To select a suitable aeration regime for the seedling culture, an experiment was conducted with 10g of fresh inoculum cultivated in a bioreactor with temporary stirring, containing 200ml of liquid nutrient medium MS (Murashiga and skoog) with growth regulators 2.0mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine and 30g/L sucrose. The experiment lasted 30 days. The accumulation of fresh biomass, phytochemical profile and antioxidant activity were monitored under 3 operating regimes – 15min. aeration/4 hours rest, 15min. aeration/8 hours rest, 15min. aeration/12 hours rest.

6. Selection of a suitable nutrient medium for cultivation of suspension cell culture of jasmine gardenia.

To conduct the experiment, fresh seed from a cell suspension culture of gardenia is used. Erlenmeyer flasks with a volume of 2 liters containing 30% fresh seed are used on a rotary shaker, at 115 revolutions per minute, at 26°C, in the dark, for 21 days. Three types of nutrient medium are used: MS (Murashiga and skoog), WP (Woody plant) and B5 with growth regulators 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine, 30g/L sucrose. Then, fresh biomass is collected, dried and extracted to determine the accumulated fresh biomass, conduct HPLC analysis, and analyze the antioxidant activity.

7. Two-factor optimization

7.1 Optimization of cell suspension cultures

For the experiment with three replicates, fresh seed cultured on Woody plant medium with growth regulators 2mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine was used.

It was cultivated in 2-liter Erlenmeyer flasks containing 30% fresh seed on a rotary shaker, at 115 rpm, at 26°C, in the dark, for 12 days. Then, fresh biomass was collected, dried and extracted for HPLC analysis, antioxidant activity analysis, and determination of somaclonal variability.

7.2 Optimization of seedling cultures

For the experiment, temporary stirring bioreactors containing 200ml of liquid nutrient medium (Murashiga and skoog) with different concentrations of salts and sucrose are used.

10g of fresh inoculum is used to start the experiment. The duration of the experiment is 30 days. After the last day, fresh biomass is collected, dried and used for the needs of determining the phytochemical profile, antioxidant activity and determining somaclonal variability.

8. Analysis of somaclonal variability

8.1 DNA extraction

100 mg of fresh biomass is used for DNA extraction. The DNeasy Plant Pro Kit (Qiagen, cat# 69204) is used strictly following the specified methodology. Quantitative and qualitative DNA was determined by spectrophotometric measurement (Biochrom WPA Biowave DNA, using BioDrop 125 CUVETTE) measuring the ratio 260/280 and 260/230 and subsequent verification of the isolated DNA on 1% gel electrophoresis. The final DNA concentration was 15 ng/μL.

8.2 SSR analysis

For the purpose of this dissertation, 11 SSR markers were selected after a literature review (Xu, Y.-Q. et al., 2015 and Denk, S-y, 2015) presented in Table **1.**

Table 1. SSR markers (Xu, Y.-Q. и др. 2015; Deng, S.-Y.; 2015)

Locus	F Primer	R Primer	Genetic bank
eGJ010	F: GAGGTGTTTGCCATCCTGGA	R: TGTTCCTCCATTTGCTGCCA	KM279434
eGJ118	F: CAACACCTTTGCTCGACTGC	R: GTCAGCACTCCACAGCATCT	KM279449
eGJ144	F: TGAGCTGATGCACTCACAGA	R: CCTGGACCTGGAGAAAGACG	KM279455
GJ04	F: GTCCAACTATCCATAAACAT	R: AGAAGAAAGAAGGAAACAGA	JQ750623
GJ08	F: GGAGCTGAGACTAAAGTAAG	R: ATCCAGAATCTAAAGCAGT	JQ750627
GJ09	F: CGGACCCAGTTCGAGAAGC	R: ATCCATCGCCTGAGCAACC	JQ750628
GJ10	F: TCACCTTTATCACTACCAT	R: GTTGACAAGTGTTGAGAATA	JQ750629
GJ16	F: ATGGAATATCATTTGAGCT	R: GTAGACGATGTCAGAAACC	JQ750635
GJ17	F: GAGATTGGAAATATGAACAC	R: CAACTCTAGGAACAAGGTA	JQ750636
GJ02	F: GGCTCTACAATCTGATTATCTT	R: ACCTCTAGCAATTCTCCAT	JQ750621

GJ03	F: CCTTTCTACCTCCTCCATA	R: ATCTGACAAGTTCCACCAA	JQ750622

9.Statistical analysis

Three independent biological samples (n=3) were used for all experiments. All spectrophotometric experiments were performed with 8 technical replicates. Results are presented as mean (n=3) and standard deviation (±SD). Correlation analysis was performed using the Pearson correlation method. Data were normalized using a factor = 1 and log 10 transformation. Statistical tests were performed using Metaboanalyst 5.0 and MiniTab 17 Statistical Software (Minitab INC, State College, PA, USA). The number of alleles per locus (Na), the effective number of alleles (Ne), heterozygosity (Ho), expected heterozygosity (He), and polymorphic information content (PIC) were calculated using the Cervus 3.0.7 software package.

III. RESULTS AND DISCUSSION

1. Obtaining *in vitro* systems

The preparation of in vitro systems used sterilized explants from the parent plant *Gardenia jasminoides* Ellis, prepared as described in materials and methods.

Figure 1. *Gardenia jasminoides* Ellis

2. Comparison of the produced primary and secondary metabolites, antioxidant activity and somoclonal variability of gardenia leaves and their derived *in vitro* systems.

2.1 Phytochemical profiling with GC/MS of biomass from *in vitro* systems and leaves of *Gardenia jasminoides* Ellis

GC/MS analysis was performed to identify the primary metabolites of the studied *in vitro* systems and the source plant gardenia.

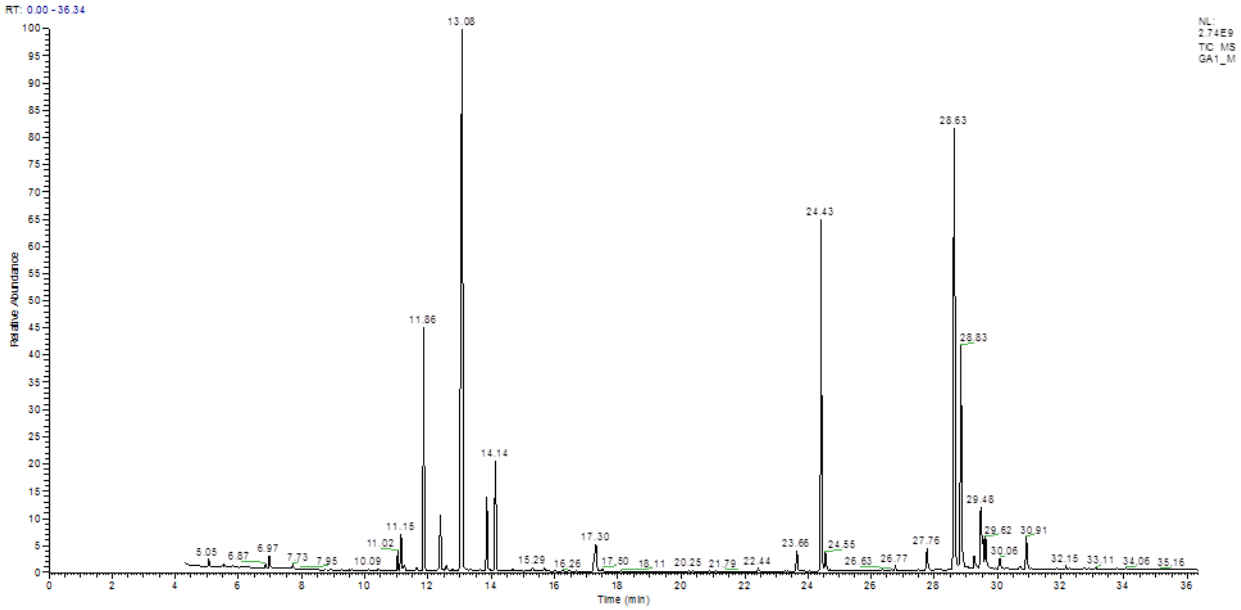


Figure 2. Chromatogram of *Gardenia jasminoides* Ellis extract

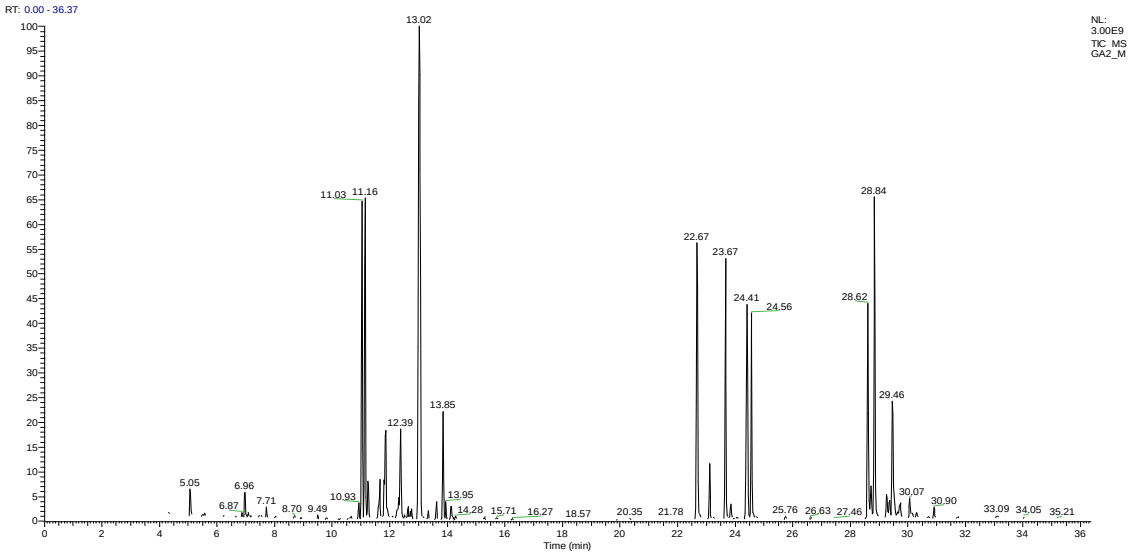


Figure 3. Chromatogram of an in vitro plant extract *Gardenia jasminoides* Ellis

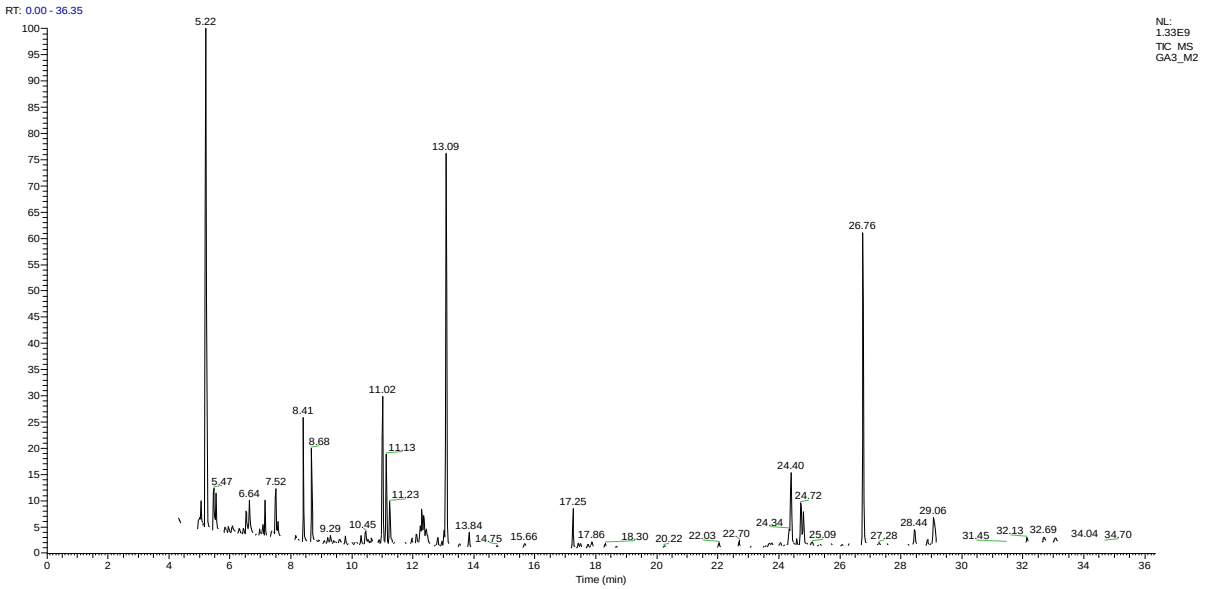


Figure 4. Chromatogram of callus extract

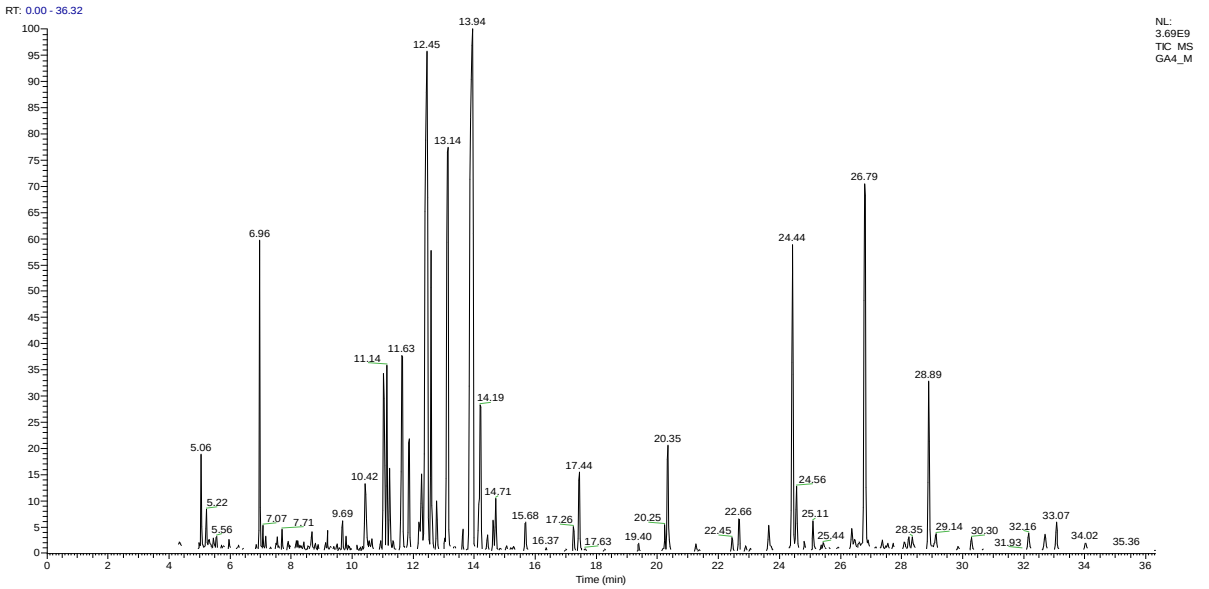


Figure 5. Chromatogram of a cell suspension extract

A total of 67 metabolites were identified in the plant and derived cultures and 41 and 34 metabolites in callus and cell suspension cultures, respectively (Figure 6). The principal component analysis performed showed that the four principal components had eigenvalues greater than unity, explaining 97.6% of the variation in the data.

Discriminant analysis (PLS-DA) was used to identify compounds that could differentiate the obtained in vitro cultures (sprouting cultures, callus and cell

suspension). Interestingly, hydrocarbons and fatty alcohols such as tetracosane, hexacosane; and 1-octadecanol were present in the highest concentrations in the seedling culture, lower in the plant and completely absent in the callus and cell suspensions.

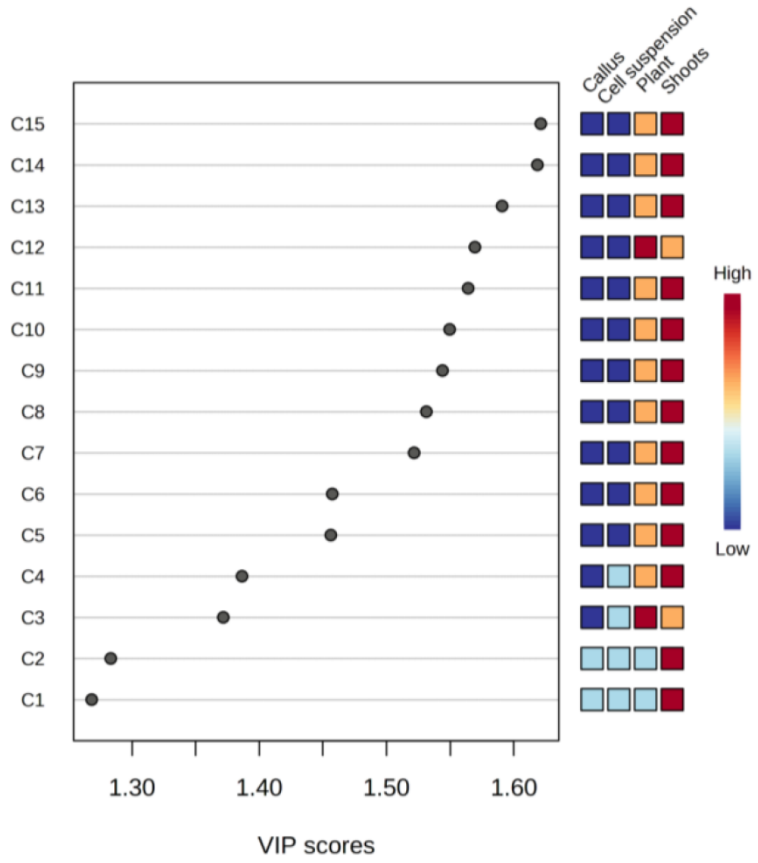


Figure 6. Variable importance in projection (VIP) scores plot (measure in PLS-DA), indicating the most discriminating compounds in descending order of importance: C1—Heptane, branched; C2—Tetradecanoic acid (methyl ester C14:0); C3—Sucrose; C4—Galactosylglycerol; C5—1-Hexacosanol; C6—2-Hydroxytricosanoic acid; C7—Arabitol; C8—Arabitol; C9—Eicosanoic acid (methyl ester, Arachidic acid, C20:0); C10—2-Hydroxy-hexacosanoic acid; C11—Octadecane, 2-methyl; C12—1-Eicosanol; C13—1-Octadecanol; C14—Hexacosane; C15—Tetracosane.

To better visualize the differences observed in the metabolite patterns of the studied samples, the relative concentrations of the 30 most significantly different metabolites

identified in the leaves of the Gardenia plant, sprout culture, callus and cell suspension cultures (determined by processing the GC/MS data with ANOVA, t-test) results are mapped in a map. The grouping of the groups clearly shows the difference between the sprout culture and the other samples (callus, cell suspension and plant leaves). The most metabolites found in the shoots are fatty acids, hydrocarbons and fatty alcohols. The callus culture is rich in phytosterols and saccharides. The cell suspension is rich in sugars and sugar acids, while in the leaves of the plant sugars, fatty and organic acids predominate (Figure 7).

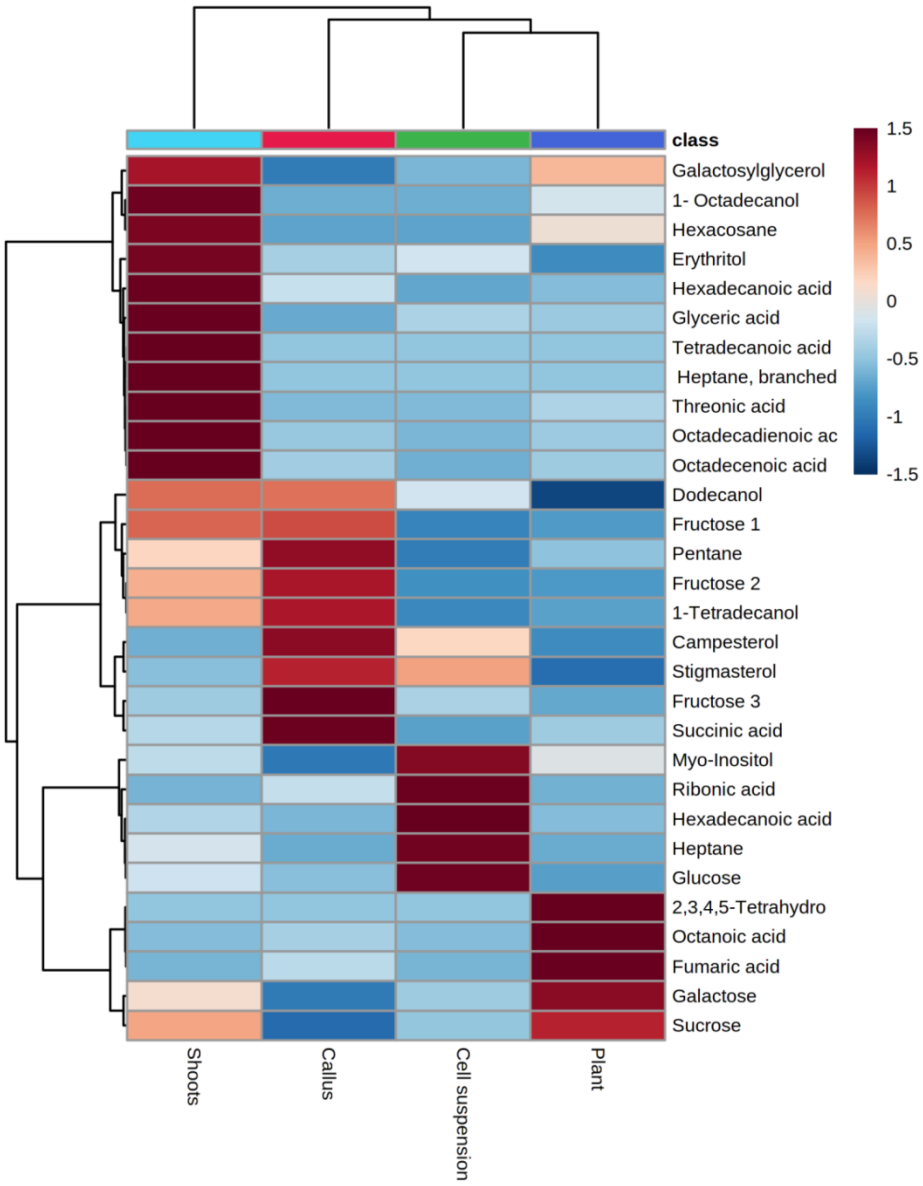


Figure 7. Hierarchically clustered heatmap of 30 most significant (T-test/ANOVA, *n* = 3) metabolites found in *G. jasminoides* plant leaves (Purple) and in vitro grown shoots (light blue), callus (red), and cell suspension (green) cultures, identified and quantified by GC/MS. Clustering was performed by using the Ward method with Euclidean distance. Each column in the Heatmap analysis represents a sample (plant, shoots, callus, and cell suspensions), and each row indicates metabolite concentrations at their highest and lowest levels (ranging from +1.5 to −1.5), coded in red and blue colors with different intensities.

2.2. Quantification of phenolic compounds in gardenia leaves and in vitro cultures by HPLC

The phenolic content (nine phenolic acids and six flavonoids) of G. jasminoides Ellis plant leaves and in vitro cultured sprouts, callus and cell suspension cultures was analyzed. The results are presented in Table 2. The differentiated tissue samples (plant leaves and sprouts) accumulated phenols in higher concentrations than those found in undifferentiated tissues (callus and cell suspension). However, there were some interesting differences. Data analyses showed that sprouts accumulated chlorogenic acid, quercetin, kaempferol and rutin in significantly higher ($p < 0.01$) amounts compared to plant leaves. Furthermore, sprouts were the only ones to accumulate protocatechuic and p-coumaric acid. Rutin is one of the major flavonoids reported in gardenia plant extracts (mainly in fruits) (Chen, L., 2020; Yin, F., 2018; Saravanakumar, K., 2021; Wang, L., 2016) and in this study was shown to accumulate in higher amounts in differentiated than in undifferentiated gardenia in vitro culture.

It is important to note that undifferentiated callus and cell suspension cultures are the only in vitro systems that produce (+)-catechin and also accumulate vanillic acid unlike plant leaves.

Table 2. Quantification of phenolic compounds in gardenia leaves and *in vitro* cultures by HPLC

Compounds	Plant, µg/g DW	Shoots, µg/g DW	Callus, µg/g DW	Cell Suspension, µg/g DW
Protocatechuic acid	ND [b,*]	8.65 ± 0.41 [a]	ND [b]	ND [b]
(+)-Catechin	ND [c]	ND [c]	28.24 ± 0.99 [b]	47.13 ± 3.40 [a]
Chlorogenic acid	173.02 ± 9.67 [b]	411.47 ± 11.67 [a]	10.21 ± 0.77 c	12.08 ± 0.27 [c]
Vanillic acid	ND [c]	375.02 ± 19.34 [a]	16.21 ± 1.03 [b]	17.48 ± 1.38 [b]
Caffeic acid	84.34 ± 9.16 [a]	97.57 ± 7.12 [a]	28.32 ± 0.95 [b]	ND [c]

Syringic acid	84.68 ± 1.34 [a]	26.85 ± 1.58 [b]	16.98 ± 0.01 [c]	14.01 ± 2.96 [c]
(−)-Epicatechin	386.83 ± 10.90 [a]	177.86 ± 4.53 [b]	48.44 ± 1.84 [c]	53.31 ± 2.86 c
p-Coumaric acid	ND [b]	15.15 ± 2.52 [a]	ND [b]	ND [b]
Ferulic acid	83.38 ± 9.93 [a]	32.64 ± 3.28 [b]	21.76 ± 2.40 [bc]	13.74 ± 1.32 [c]
Salicylic acid	1344.97 ± 100.11 [a]	1262.82 ± 193.99 [a]	10.67 ± 3.37 [b]	9.78 ± 1.09 [b]
Rutin	40.75 ± 1.50 [a]	50.35 ± 9.59 [a]	3.18 ± 0.40 [b]	3.06 ± 0.07 [b]
Hesperidin	309.32 ± 13.40 [a]	233.42 ± 46.31 [b]	ND [c]	ND [c]
Rosmarinic acid	1636.22 ± 135.45 [a]	419.72 ± 101.73 [b]	31.27 ± 6.50 [c]	32.30 ± 1.98 [c]
Quercetin	39.33 ± 2.45 [b]	284.28 ± 22.85 [a]	0.12 ± 0.02 [c]	ND [c]
Kaempferol	4.15 ± 1.63 [b]	286.05 ± 24.56 [a]	ND [c]	ND [c]

ND—Not Detected. The presented values are Means ± SD, $n = 3$. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

2.3 Antioxidant activity of phenolic extracts of gardenia leaves and in vitro cultures

The antioxidant activities of methanol extracts of leaves, sprouts, callus and cell suspension cultures of G. jasminoides were analyzed using four reliable in vitro assays. The results are presented in Table 3. The leaf extract of the plant showed the highest total phenolic content (4.05 ± 0.36 mg GAE/g WB), followed by the extracts of sprouts, cell suspension and callus cultures. The antioxidant potential of extracts from undifferentiated in vitro cultures (callus and cell suspension) was significantly lower ($p < 0.01$) compared to the leaves of the plants and differentiated shoots.

Table 3. Antioxidant activity of phenolic extracts of gardenia leaves and *in vitro* cultures

	Plant	Shoot culture	Callus	Cell suspension
Cu-(II)-reducing activity, µM TE/g dry biomass	349.74 ± 39.39 [a]	281.28 ± 17.34 [b,*]	49.56 ± 8.50 [c,*]	53.45 ± 8.41 [c,*]
Fe-(III)-reducing activity, µM TE/g dry biomass	251.68 ± 16.45 [a]	234.56 ± 15.59 [a,*]	5.47 ± 0.65 [b,*]	10.95 ± 0.50 [b,*]
DPPH-radical scavenging activity, µM TE/g dry biomass	368.41 ± 41.77 [b]	647.21 ± 33.29 [a,*]	8.73 ± 1.50 [c,*]	18.29 ± 2.69 [c,*]
ABTS radical scavenging activity, µM TE/g dry biomass	2162.79 ± 62.62 [a]	1394.90 ± 15.00 [b,*]	12.50 ± 1.72 [c,*]	23.57 ± 2.09 [c,*]
Total polyphenols, µM GAE/g dry biomass	4.05 ± 0.36 [a]	3.03 ± 0.20 [b,*]	0.18 ± 0.02 [c,*]	0.27 ± 0.01 [c,*]

* ND—Not Detected. The presented values are Means ± SD, $n = 3$. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

2.4 Somaclonal Variability in Gardenia and the In Vitro Cultures Obtained

The data show that all SSRs, except GJ08, have more than three alleles and can be used to assess genetic diversity in the studied Gardenia systems. The number of detected alleles (Na) ranged from 2 to 6 with an average of 5.636 ± 2.157 alleles per locus, while the effective number of alleles (Ne) ranged from 1 to 4 with an average of 3.0 ± 1.095. These values are significantly higher than those reported for Gardenia somaclones (average Na = 1.85, average Ne = 1.85) (Ebrahim, M.A., 2022), indicating that the differentiated and undifferentiated Gardenia in vitro cultures used in this study have much higher genetic variation. This is also confirmed by the high values of observed heterozygosity (Ho = 1) and expected heterozygosity (He = 0.972 ± 0.051), which is clear evidence of high genetic variability among the studied systems.

A cluster analysis (Figure 8) of the SSR data was performed in order to group the gardenia plant and the resulting in vitro cultures into clusters using Euclidean distance similarity coefficients. The highest genetic similarity (35.47) was found between the plant and the cell suspension culture, while the similarities between the plant and the

callus (13.53) and the plant and the seedling culture (−7.09) were significantly lower. Analyzing the data, it can be assumed that the significant differences observed in the metabolic profile of the differentiated seedling culture correlate with the observed high genetic distance compared to the Gardenia plant and undifferentiated in vitro cultures (callus and cell suspensions). This is an interesting observation, as it is usually postulated that differentiated in vitro cultures show less genetic variability than undifferentiated ones (Steingroewer, J., 2013).

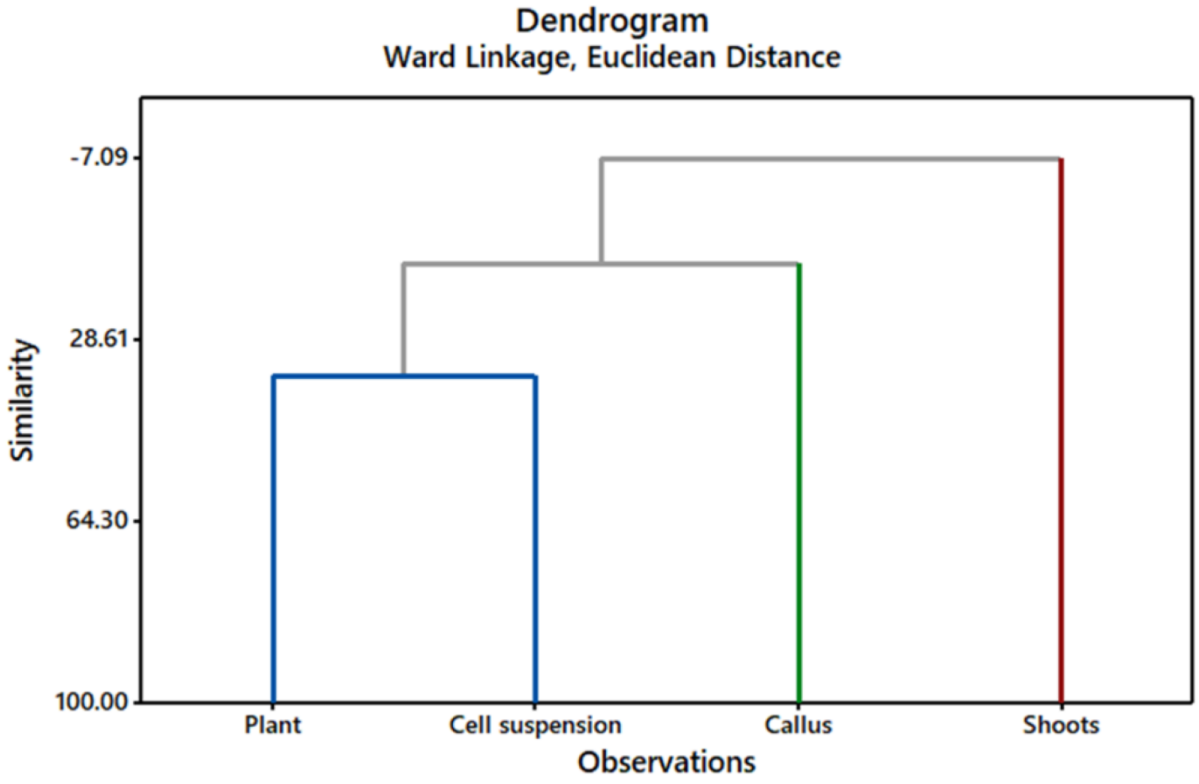


Figure 8. Dendrogram constructed using SSR data and Ward's clustering method with Euclidean distance. The dendrogram shows genetic similarities between *Gardenia* plant, differentiated shoots and undifferentiated callus, and cell suspension cultures. Cultures having different genetic similarities were grouped in clusters with different colors (cluster 1—blue, cluster 2—green, and cluster 3—red)

3. Gardenia callus culture growing on different nutrient media

To monitoring the changes in dry biomass production, phytochemical profile, antioxidant activity and genetic stability in the obtained in vitro callus culture, cultivation on different nutrient media and different light regimes was carried out (Figure 9).

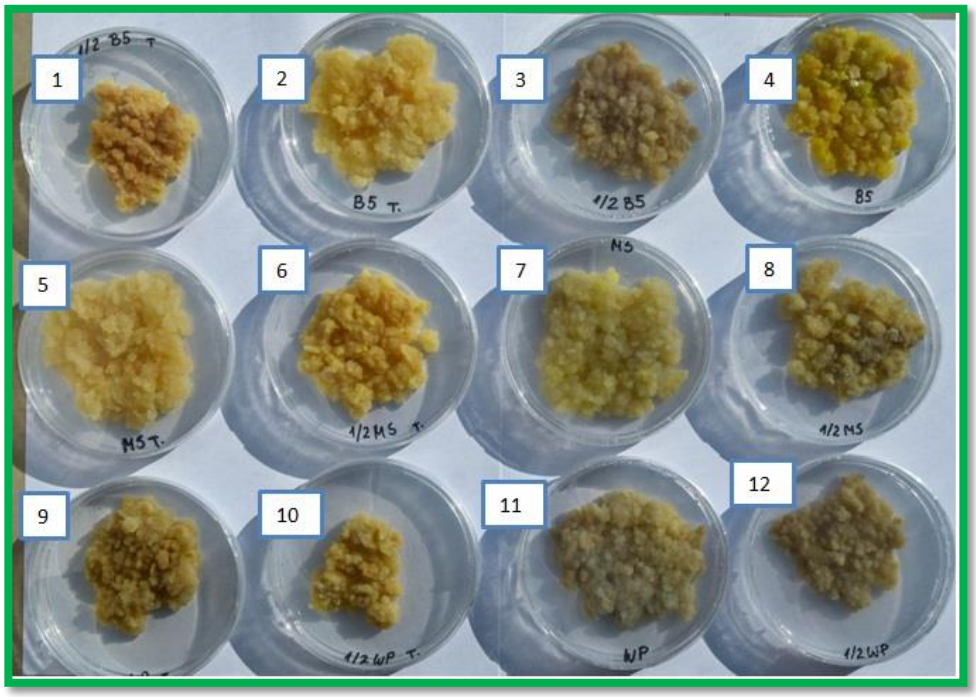


Figure 9. Callus cultures of jasmine gardenia on different nutrient media and light regimes 1- ½ B5 in the dark; 2 - B5 in the dark; 3 - ½ B5 light (16/8); 4- B5 light (16/8); 5 - MS in the dark; 6 - ½ MS in the dark; 7 - MS light (16/8); 8 - MS light (16/8); 9 – WP in the dark; 10 – ½ WP in the dark; 11 – WP light (16/8); 12 – ½ WP light (16/8)

Optimal light intensity and selective wavelength enhance diosgenin production in callus cultures of Dioscorea deltoidea (Ahmad, N. et al., 2016). However, physiological and morphological responses of plants to light quality are significantly varied depending on the plant species (Ahmad, N. et al., 2016). Tariq and her team (Tariq, U., et al. 2014) reported significant variation in both morphogenic and biochemical processes in callus cultures of Artemisia absinthium exposed to different

monochromatic lights. It is a well-known fact that light plays a key role in primary and secondary metabolism and various developmental processes of plants (Ahmad, N. et al., 2016). However, for the first time, in the present work, the effect of light on the production of secondary metabolites by callus culture of gardenia is monitored and its key influence is proven.

3.1 Analysis of changes in the synthesis of secondary metabolites

In recent years, various studies have been conducted to prove the biological potential of the plant and its ability to produce active metabolites. So far, there is no data available on how light and the composition of the nutrient medium affect the production of metabolites in callus culture of jasmine gardenia.

In the present work, we analyze how the phytochemical profile of callus cultures changes under different light regimes - in the light and in the dark, and different nutrient medium bases (Murashiga and skoog, Woody plant, B5). The results of the experiment are presented sequentially in Table 4, Table 5, Table 6 and Table 7, and show us that there are significant differences in the production of secondary metabolites when cultivating the cultures on a full nutrient medium base and on ½ of the nutrient medium base, cultivated in the light and in the dark.

Table 4. HPLC quantification of phenolic compounds of callus cultured on ½ MS, ½WP, ½B5 medium with 16/8 light regime

	1/2 MS - 16/8	1/2 WP - 16/8	1/2 B5 - 16/8
Chlorogenic acid, µg/g	11,33 ± 3,07^{вb}	61,24 ± 14,04^{a}	25,25 ± 2,25^{б}
Caffeic acid, µg/g	3,05 ± 1,01^{b}	2,90 ± 0,7^{b}	21,91 ± 0,01^{a}
Ferulic acid, µg/g	6,68 ± 1,95^{a}	5,07 ± 0,1^{a}	4,37 ± 0,11^{a}
Rutin, µg/g	ND	13,74 ± 8,43^{a}	ND
Rosmarinic acid, µg/g	77,22 ± 6,15^{a}	55,69 ± 6,13^{b}	34,81 ± 2,69^{b}
Quercetin, µg/g	1,12 ± 0,07^{c}	14,34 ± 0,45^{a}	9,52 ± 1,75^{б}
kaempferol, µg/g	ND	1,22 ± 0,31^{a}	1,44 ± 0,05^{a}
Catechin, µg/g	ND	36,98 ± 3,87^{a}	27,85 ± 15,97^{a}
Vanillic acid, µg/g	71,02 ± 1,71^{a}	58,56 ± 10,58^{б}	ND
Syringic acid, µg/g	ND	24,03 ± 6,49^{a}	7,94 ± 0,31^{b}
Epicatechin, µg/g	ND	98,47 ± 6,87^{a}	71,92 ± 36,25^{b}
p-Coumaric acid, µg/g	ND	14,80 ± 1,35^{a}	4,03 ± 3,77^{a}

Salicylic acid, µg/g	12,91 ± 6,58[b]	25,44 ± 5,43[a]	ND
Hisperidin, µg/g	3,11 ± 1,2[b]	11,41 ± 0,02[a]	ND

* ND—Not Detected. The presented values are Means ± SD, $n = 3$. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

Table 5. HPLC quantification of phenolic compounds of callus cultured on MS, WP, B5 medium with 16/8 light regime

	MS - 16/8	**WP - 16/8**	**B5 - 16/8**
Chlorogenic acid, µg/g	32,97 ± 2,35[b]	172,02 ± 8,19[a]	3,23 ± 9,57[в]
Caffeic acid, µg/g	1,37 ± 0[b]	15,71 ± 0,75[a]	6,12 ± 3,55[б]
Ferulic acid, µg/g	7,17 ± 0,8[b]	9,30 ± 3,01[a]	5,22 ± 0,22[b]
Rutin, µg/g	ND	6,46 ± 0,55[a]	ND
Rosmarinic acid, µg/g	29,44 ± 1,24[c]	217,29 ± 5,51[a]	89,19 ± 4,18[b]
Quercetin, µg/g	2,28 ± 0,023[b]	20,53 ± 4,12[a]	7,42 ± 0,52[b]
kaempferol, µg/g	3,95 ± 0,18[b]	11,96 ± 0,92[a]	10,84 ± 6,81[a]
Catechin, µg/g	ND	37,67 ± 12,15[a]	ND
Vanillic acid, µg/g	22,14 ± 0,6[c]	139,61 ± 3,92[a]	44,99 ± 6,16[b]
Syringic acid, µg/g	12,81 ± 0,89[b]	57,57 ± 3,68[a]	11,65 ± 0,5[b]
Epicatechin, µg/g	41,53 ± 4,96[b]	101,61 ± 24,60[a]	22,13 ± 4,08[c]
p-Coumaric acid, µg/g	ND	25,53 ± 2,18[a]	5,71 ± 7,31[b]
Salicylic acid, µg/g	25,91 ± 0,9[b]	45,20 ± 2,62[a]	19,22 ± 4,41[b]
Hisperidin, µg/g	4,09 ± 0,14[b]	23,77 ± 0,05[a]	ND

* ND—Not Detected. The presented values are Means ± SD, $n = 3$. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

Comparing the results of the obtained metabolites of the callus cultures cultivated in the dark, we will notice that we have higher values in the cultures cultivated on WP medium (Table 6 and Table 7). The value of chlorogenic acid of the callus culture cultivated in the dark is 18.68 ± 2.19 µg/g (Table 7) for comparison on the same WP medium, but cultivated in the light, the callus produces 172.02 ± 8.19 µg/g chlorogenic acid (Table 11).

The results obtained for the concentration of chlorogenic acid were statistically indistinguishable, 2.10 ± 1.37 µg/g cultivated on ½ MS in the dark and 2.86 ± 0.21 µg/g cultivated on ½ B5 in the dark (Table 6), compared to the same nutrient media but cultivated in the light, the callus produced 11.33 ± 3.07 µg/g and 25.25 ± 2.25 µg/g of chlorogenic acid, respectively (Table 4). The highest concentration of chlorogenic acid from the callus cultures cultivated in the dark was found in nutrient medium WP 18.68 ± 2.9 µg/g, while only 5.41 ± 0.9 µg/g of chlorogenic acid was found in ½ WP

in the dark (Table 6 and Table 7). For comparison of the same nutrient media but in the light, the callus produced 172.02 ± 8.19 µg/g and 61.24 ± 14.04 µg/g, respectively (Table 4 and Table 5).

The results show that light is a major physical factor that, in a controlled environment, plays a direct role in the production of secondary metabolites, both by the plant and by the callus cultures obtained from it.

The tendency for a greater number of metabolites produced by callus cultures cultivated in the light, compared to those cultivated in the dark, is also observed in nutrient medium B5.

Table 6. HPLC quantitative determination of phenolic compounds of callus cultivated on 1/2 MS, 1/2WP, 1/2B5 nutrient medium in the dark

	1/2 MS тъмно	1/2WP тъмно	1/2 B5 тъмно
Chlorogenic acid, µg/g	2,10 ± 1,37[b]	5,41 ± 0,91[a]	2,86 ± 2,21[b]
Caffeic acid, µg/g	2,88 ± 0,9[b]	8,63 ± 7,44[a]	1,34 ± 0,08[b]
Ferulic acid, µg/g	5,60 ± 0,2[a]	4,72 ± 0,09[a]	5,72 ± 3,15[a]
Rutin, µg/g	ND	2,85 ± 0,18[a]	ND
Rosmarinic acid, µg/g	5,87 ± 0,44[a]	4,86 ± 0,37[a]	ND
Quercetin, µg/g	ND	4,48 ± 2,41[a]	1,72 ± 21[b]
kaempferol, µg/g	0,94 ± 0,08[c]	ND	1,29 ± 0,5[b]
Catechin, µg/g	ND	ND	7,88[б]
Vanillic acid, µg/g	27,97 ± 0,49[a]	ND	18,36 ± 9,73[b]
Syringic acid, µg/g	ND	21,02 ± 0,16[a]	ND
Epicatechin, µg/g	ND	15,27 ± 2,08[a]	ND
p-Coumaric acid, µg/g	2,68 ± 0,03[b]	15,19 ± 7,15[a]	ND
Salicylic acid, µg/g	19,13 ± 0,33[b]	27,50 ± 0,12[a]	15,59 ± 4,3[b]
Hisperidin, µg/g	14,67 ± 2,98[a]	0,55 ± 0,21[b]	Няма

* ND—Not Detected. The presented values are Means ± SD, $n = 3$. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

Table 7. HPLC quantification of phenolic compounds of callus cultured on MS, WP, B5 medium in the dark

	MS тъмно	WP тъмно	B5 тъмно
Chlorogenic acid, µg/g	5,36 ± 0,05[c]	18,68 ± 2,19[a]	15,99 ± 5,19[b]
Caffeic acid, µg/g	ND	2,33 ± 0,3[a]	3,70 ± 2,35[a]
Ferulic acid, µg/g	3,44 ± 0,13[b]	16,85 ± 2,19[a]	6,10 ± 0,43[b]
Rutin, µg/g	ND	0,93 ± 0,03[a]	ND
Rosmarinic acid, µg/g	12,84 ± 0,12[b]	15,81 ± 6,18[a]	6,99 ± 0,63[c]
Quercetin, µg/g	19,47 ± 10[a]	14,36 ± 9,66[b]	ND
kaempferol, µg/g	0,50 ± 0,08[b]	ND	14,80 ± 0,16[a]

Catechin, µg/g	ND	12,65 ± 0,47[a]	1,59 ± 0,84[a]
Vanillic acid, µg/g	5,51 ± 0,38[a]	ND	ND
Syringic acid, µg/g	ND	22,21 ± 2,68[a]	ND
Epicatechin, µg/g	ND		ND
p-Coumaric acid, µg/g	2,38 ± 0,04[b]		ND
Salicylic acid, µg/g	14,53 ± 1,42[b]	21,72 ± 5,31[a]	17,76 ± 2,60[b]
Hisperidin, µg/g	ND	25,07 ± 4,41[a]	2,45 ± 2,05[b]

* ND—Not Detected. The presented values are Means ± SD, $n = 3$. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

3.2 Antioxidant activity

After monitoring the changes in the phytochemical profile of the cultures, the antioxidant activity was also monitored by 4 methods - Table 8, Table 9, Table 10, Table 11.

The results shown in the tables confirm the trend from analyzing the phytochemical profile, and here the highest antioxidant activity is observed in the cultures cultivated on WP nutrient medium in the light, compared to those in the dark.

Таблица 8. Антиоксидантна активност на калусни култури култивирани на хранителни среди MS, 1/2MS, WP, 1/2WP, B5, 1/2B5 съответно на режим на светлина 16/8 и на тъмно по метода DPPH

	DPPH, µM TE/g СБ		**DPPH, µM TE/g СБ**
1/2 MS 16/8	7,22 ± 3,89[a]	**MS 16/8**	5,02 ± 0,3[b]
1/2 WP 16/8	3,82 ± 0,8[b]	**WP 16/8**	5,86 ± 0,8[a]
1/2 B5 16/8	4,50 ± 0,31[a]	**B5 16/8**	3,57 ± 0,04[a]
1/2 MS in the dark	4,06 ± 1,3[a]	**MS in the dark**	2,93 ± 0,07[b]
1/2 WP in the dark	3,62 ± 1,4[a]	**WP in the dark**	3,71 ± 0,44[a]
1/2 B5 in the dark	3,85 ± 0,3[a]	**B5 in the dark**	3,51 ± 0,2[a]

* ND—Not Detected. The presented values are Means ± SD, $n = 3$. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

The radical scavenging activity of the callus culture towards the ABTS radical has the greatest potential when the culture is grown on WP medium in the light (Table 9).

Таблица 9. Антиоксидантна активност на калусни култури култивирани на хранителни среди MS, 1/2MS, WP, 1/2WP, B5, 1/2B5 съответно на режим на светлина 16/8 и на тъмно по метода TEAC

	TEAC, μM TE/g СБ		TEAC, μM TE/g СБ
1/2 MS 16/8	$1,21 \pm 0,5^{a}$	**MS 16/8**	$0,24 \pm 0,02^{b}$
1/2 WP 16/8	$3,95 \pm 1,74^{b}$	**WP 16/8**	$6,08 \pm 0,59^{a}$
1/2 B5 16/8	$4,79 \pm 0,44^{a}$	**B5 16/8**	$1,69 \pm 0,44^{b}$
1/2 MS in the dark	$0,29 \pm 0,03^{b}$	**MS in the dark**	$0,99 \pm 0,02^{a}$
1/2 WP in the dark	$1,76 \pm 0,3^{a}$	**WP in the dark**	$1,05 \pm 0,39^{b}$
1/2 B5 in the dark	$4,44 \pm 0,15^{a}$	**B5 in the dark**	$0,64 \pm 0,33^{b}$

* ND—Not Detected. The presented values are Means ± SD, $n = 3$. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

The potential for reducing copper ions is highest in cultures cultivated at ½ WP in the light. It is noted that with this method for analyzing antioxidant activity, the results obtained for callus cultures cultivated on nutrient medium B5 (Table 10).

Таблица 10. Антиоксидантна активност на калусни култури култивирани на хранителни среди MS, 1/2MS, WP, 1/2WP, B5, 1/2B5 съответно на

	CUPRAC, μM TE/g СБ		CUPRAC, μM TE/g СБ
1/2 MS 16/8	$30,24 \pm 1,88^{a}$	**MS 16/8**	$23,30 \pm 2,9^{b}$
1/2 WP 16/8	$43,73 \pm 3,25^{a}$	**WP 16/8**	$22,30 \pm 7,4^{b}$
1/2 B5 16/8	$10,20 \pm 1,55^{a}$	**B5 16/8**	$9,83 \pm 0,78^{a}$
1/2 MS in the dark	$15,52 \pm 1,77^{a}$	**MS in the dark**	$14,77 \pm 1,06^{a}$
1/2 WP in the dark	$16,32 \pm 2,24^{б}$	**WP in the dark**	$22,30 \pm 7,46^{a}$
1/2 B5 in the dark	$22,59 \pm 8,08^{a}$	**B5 in the dark**	$16,76 \pm 1,22^{b}$

The study of antioxidant activity using a method for monitoring the reduction of iron ions shows that the best activity is shown by callus cultures cultivated on WP 16/8 (Table 11).

Table 11. Antioxidant activity of callus cultures cultivated on nutrient media MS, 1/2MS, WP, 1/2WP, B5, 1/2B5 respectively under 16/8 light regime and in the dark by the FRAP method

	FRAP, μM TE/g СБ		FRAP, μM TE/g СБ
1/2 MS 16/8	$1,85 \pm 0,33^{b}$	**MS 16/8**	**$10,74 \pm 0,3^{a}$**

1/2 WP 16/8	8,40 ± 0,64[b]	**WP 16/8**	**12,17 ± 0,96[a]**
1/2 B5 16/8	5,96 ± 0,3[a]	**B5 16/8**	**4,15 ± 0,5[b]**
1/2 MS in the dark	2,20 ± 0,4[a]	**MS in the dark**	**1,27 ± 0,15[b]**
1/2 WP in the dark	6,05 ± 0,09[a]	**WP in the dark**	**5,81 ± 0,4[a]**
1/2 B5 in the dark	4,41 ± 0,33[a]	**B5 in the dark**	**2,35 ± 0,34[b]**

* ND—Not Detected. The presented values are Means ± SD, $n = 3$. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

3.3 Analyzing the changes in the genetic stability of callus cultures in relation to the plant from which they were obtained

A cluster analysis was carried out (Figure 10) in order to group the callus cultures cultivated on different nutrient media and developing respectively on the 16/8 light regime and in the dark, in relation to the plant from which they were obtained. The figure shows two large clusters, in one of which the gardenia plant is grouped, together with the callus cultures cultivated respectively on ½ WP in the dark, ½ WP in the light, WP in the dark and WP in the light. The lowest genetic variability in this cluster is between the plant and the callus cultivated on ½ WP in the dark and ½ WP in the light (3.02), and the highest is between the plant and the callus cultivated on WP nutrient medium in the light (8.54). The largest difference occurred between the gardenia plant and the callus culture cultivated on B5 nutrient medium in the dark (12.82).

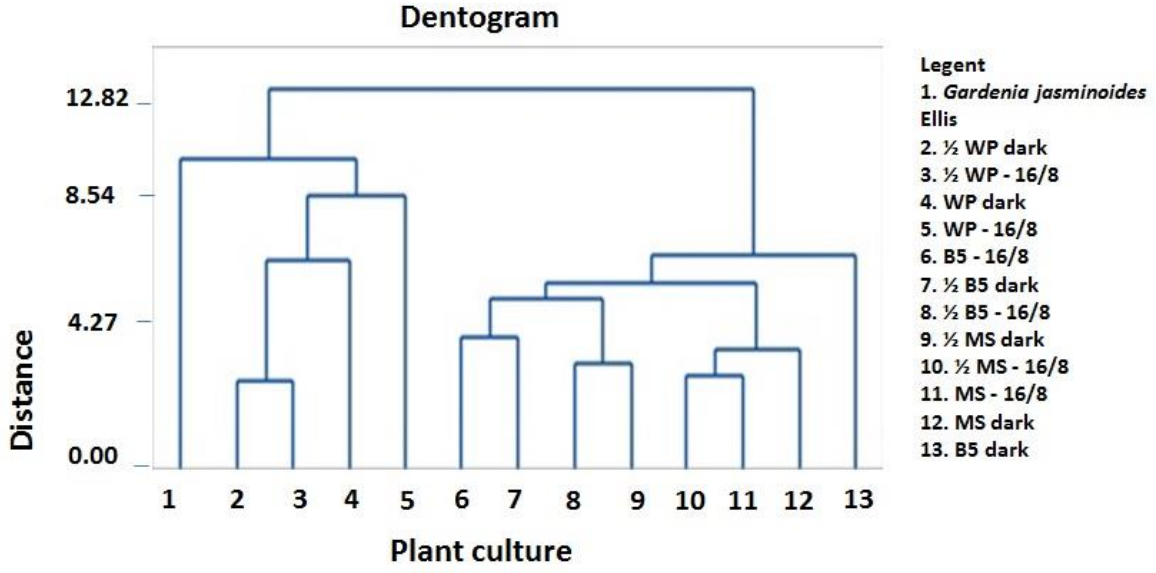


Figure 10. Cluster analysis of callus cultures cultivated on different nutrient media and growing under 16/8 light regime and in the dark, respectively, in relation to the plant from which they were obtained.

Due to the heterogeneous population of typical dedifferentiated cells that comprise calluses, the selection of a highly productive cell line is an essential step in the creation of profitable production platforms for natural products. Since the accumulation of the desired metabolites in plants is genotype-specific, the selection of appropriate species and subsequently plant parts for callus generation is crucial (Murthy HN, et al. 2014). Furthermore, differences in ploidy are associated with changes in gene expression, with higher ploidy generally appearing to result in downregulation of a large number of genes (Baebler Š., et al., 2005). The culture changes resulting from these genetic changes usually require periodic screening to maintain the desired characteristics of a given culture system (Baebler Š., et al., 2005). In this context, the application of a selectable marker in plant cells in culture has been used to circumvent this problem, maintaining highly efficient cells for more than 12 months (Raven N., et al., 2015). Therefore, it is important to optimize the culture conditions of the cultures, the changes arising from this, and it is important to track the genetic changes during cultivation and to what extent the resulting in vitro cultures differ from the parent plant, and what these changes are due to.

Information on such type of analyses on the gardenia plant and its in vitro cultures is not found in the database.

4. Selection of appropriate culture medium for cell suspension culture and seedling culture of *Gardenia jasminoides* Ellis. Dynamics. Optimization

4.1 Selection of appropriate culture medium for cell suspension culture and seedling culture of *Gardenia jasminoides* Ellis

Optimization of plant-based production systems requires more attention and flexibility in the process. In this context, promising new strategies are emerging that have good future applications. For example, monitoring cell biomass during cultivation can be an important step to maximize yield and product quality, while improving the nutrients in

the culture (Holland T.,2013). Also, culture parameters, including phytohormones and media components levels, can be optimized more quickly by using a statistical approach to design experiments (Vasilev N., 2013). Therefore, experimental design strategies, combined with line monitoring, can be very useful during the early steps in screening and optimization, to establish high-yielding cell lines. In the present work, a process optimization is performed for both cell suspension cultures (obtained from callus culture) and seedling cultures. The changes in the somoclonal variability of the cultures during the growth dynamics as well as during optimization are monitored. The aim is to determine whether the duration of cultivation has a greater impact on genetic variation, or the change in the concentration of basic nutritional components such as sucrose and salts.

4.1.1 Accumulation of dry biomass

In Figure 11, it is clearly seen that statistically indistinguishable in terms of growth are the results obtained when cultivating on nutrient media MS ((2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine) and WP (2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine), respectively 0.98 ± 0.01 g/L and 0.85 ± 0.2 g/L. and the lowest is the accumulated dry biomass when cultivating on nutrient medium B5 (Figure 11).

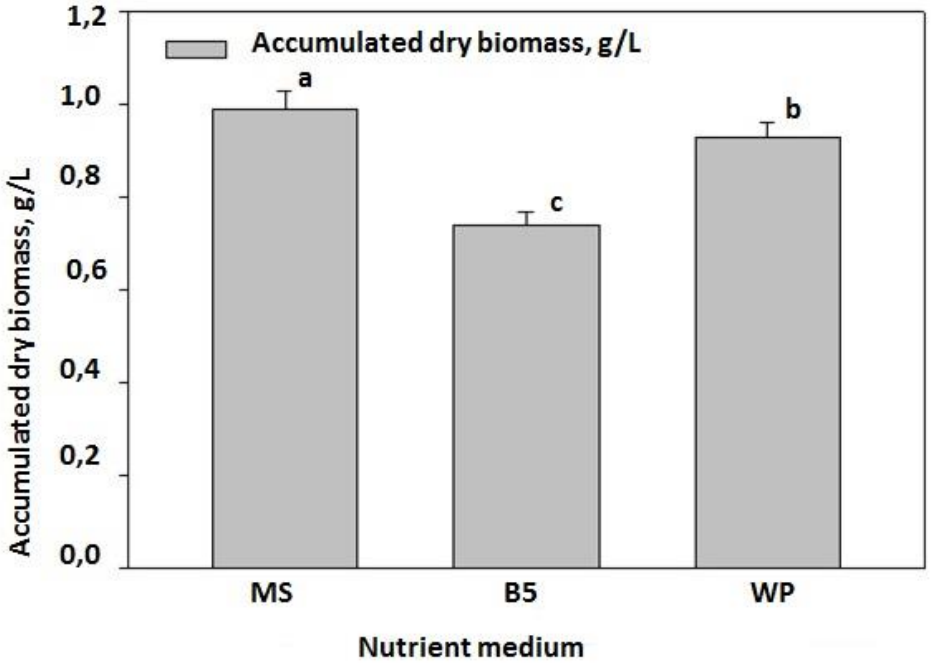


Figure 11. Accumulated dry biomass

4.1.2 Determination of secondary metabolites synthesized during cultivation on different nutrient media

Analyzing the obtained results presented in Table 12, the highest concentrations of the analyzed metabolites were found in the cell suspension culture cultivated on nutrient medium WP 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine. The statistical analysis performed showed that there were significant differences in the production of metabolites such as catechin (34.44 ± 2.46 µg/g), chlorogenic acid (20.26 ± 3.68 µg/g), epicatechin (84.44 ±7.06 µg/g), ferulic acid (27.72 ± 2.28 µg/g), salicylic acid (22.72 ± 0.79 µg/g) and rutin (45.90 ± 0.26 µg/g) on the WP 2NAA 0.5 BAP medium compared to the same metabolites on the MS 2 NAA 0.5 BAP medium.

It is striking that despite the lower result for dry biomass accumulation in nutrient media WP 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine and B5 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine, a number of metabolites such as catechin, chlorogenic acid, syringic acid and rosmarinic acid were statistically indistinguishable when compared.

In general, plant cell growth and secondary metabolite production are often inversely proportional. Thus, the production of metabolites usually occurs in the late stationary phase and is associated with growth inhibition and the production of secondary metabolism enzymes (Bhojwani SS., et al. 2013). This could explain the weaker growth on B5 medium, but the accumulation of secondary metabolites being in concentrations close to those when cultivated on WP medium where growth and dry biomass production is better.'

Table 12. Polyphenol content of cell suspension culture cultivated on different nutrient media

	MS, µg/g	WP, µg/g	B5, µg/g
(+)-Catechin	16,54 ± 19,47[b]	**34,44 ± 2,46[a]**	34,08 ± 0,78[a]
Chlorogenic acid	9,89 ± 0,91[b]	**20,26 ± 3,68[a]**	16,34 ± 3,49[a]

Vanillic acid	ND	**19,76 ± 2,16**[a]	TRACE
Caffeic acid	13,43± 0,01[b]	13,32 ± 6,88[b]	27,78 ± 9,14[a]
Syringic acid	21,68 ± 5,93[a]	18,82 ± 4,56[b]	18,89± 0,9[b]
(-)-Epicatechin	51,56 ± 13,31[b]	**70,44 ± 7,06**[a]	68,32 ± 13,67[b]
Ferrulic acid	11,45± 0,65[b]	**27,72 ± 2.28**[a]	13,16 ± 2,01[b]
Salicylic acid	11,43 ± 1,9[b]	**22,72 ± 0,79**[a]	13,93 ± 1,19[b]
Rutin	TRACE	**45,90 ± 0,26**[a]	ND
Rosmarinic acid	ND	**43,45± 0,35**[a]	**46,94 ± 0,12**[a]
Quercetin	**54,28 ±0,6**[a]	**39,29 ± 0,23**[a]	ND

* ND—Not Detected. The presented values are Means ± SD, $n = 3$. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

In the present work, we aim to find the optimal conditions for cultivating the suspension culture, both in terms of dry biomass production and polyphenol content, therefore, for the next stage of the experiment, namely monitoring the growth dynamics of the culture, we choose to work with WP nutrient medium.

4.2 Development dynamics

4.2.1 Accumulated dry biomass and secondary metabolites

Analyzing figure 12 depicting the accumulation of dry biomass by days of development of the cell suspension culture, it is clearly seen that by day 9 of the culture development, the produced dry biomass increases and reaches its maximum of 4.44 ± 0.1 g/L. After this day, it begins to decrease, with the lowest results obtained on days 15 and 18 being statistically indistinguishable, respectively 2.85 ± 0.02 g/L and 2.81 ± 0.1 g/L, or two times less than on the 9th day of cultivation.

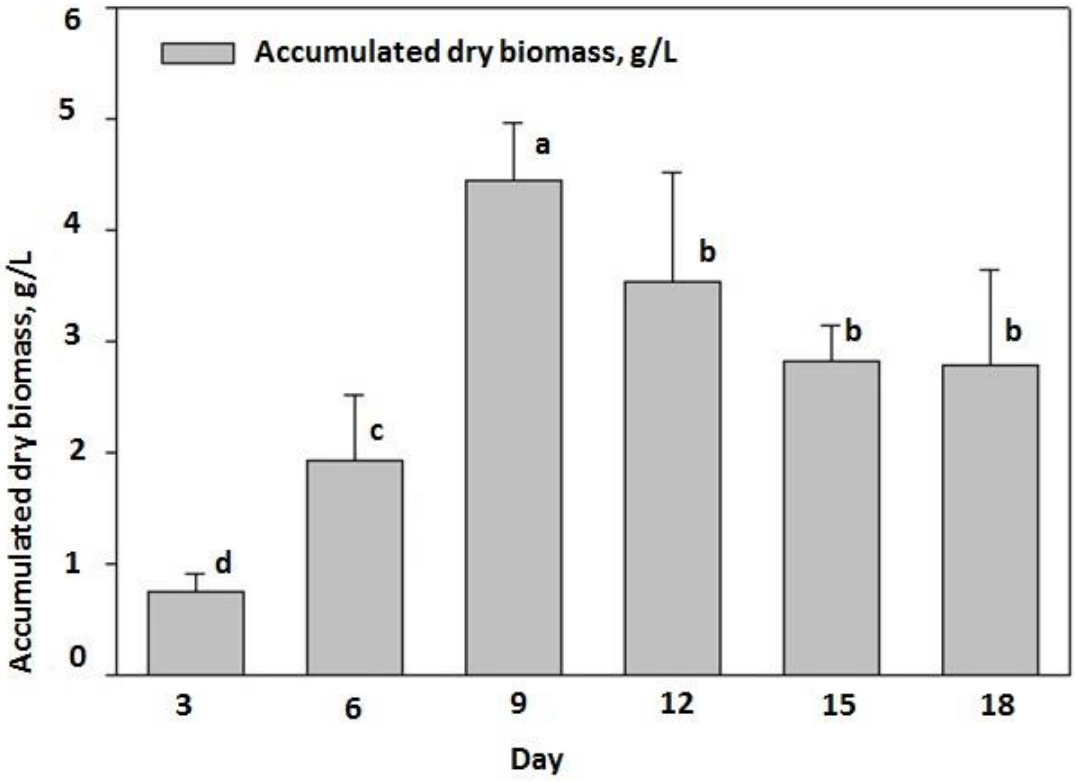


Figure 12. Accumulation of dry biomass

Regarding the accumulation of polyphenolic compounds, the data in Table 13 show that the highest concentrations of the analyzed metabolites are reached on the 9th day of cultivation. The concentrations of the metabolites are lower on the 12th and 15th days of cultivation, compared to the 9th day, but at the same time they are statistically indistinguishable from each other. This could be explained by the beginning of the aging processes of the cells in the cell suspension culture, and the lower potential for the production of polyphenolic compounds.

Table 13. Polyphenol content of cell suspension culture development dynamics

	Day 3, µg/g	Day 6, µg/g	Day 9, µg/g	Day 12, µg/g	Day 15, µg/g	Day 18, µg/g
Chlorogenic acid	32,03 ± 0^{b}	ND^{c}	26,41 ± 0^{b}	42,86 ± 0^{a}	37,67 ± 0^{a}	ND^{c}
Caffeic acid	6,42 ± 2,23^{a}	4,94 ± 3,26^{b}	2,45 ± 0,15^{c}	7,12 ± 6,33^{a}	2,81 ± 1,04^{c}	2,16 ± 0,16^{c}
Ferulic acid	13,47 ± 6,1^{b}	16,61 ± 5,72^{b}	29,11 ± 4,51^{a}	5,88 ±0,09^{c}	5,83 ± 0,28^{c}	6,69 ± 2,73^{c}
Rosmarinic acid	14,26,86 ± 4,4^{c}	28,96 ± 6,99^{b}	36,51 ± 5,81^{a}	26,17 ± 30,17^{b}	21,14 ± 60,66^{b}	13,75 ± 17,83^{c}

Quercetin	20,06 ± 8,69^{b}	18,49 ± 5,34^{b}	34,78 ± 24,37^{a}	25,01 ±2,84^{b}	11,46 ± 6,01^{c}	5,26 ± 1,7^{c}
Kaempferol	4,42 ± 0,22^{c}	7,28 ± 2,42^{b}	7,40 ± 0,64^{b}	10,29 ± 5^{a}	13,78 ± 0^{a}	8,34 ± 1,04^{b}
Gallic acid	ND^{c}	8,77 ± 0^{a}	10,79 ±0^{a}	11,69 ± 1^{a}	7,44 ± 0^{b}	ND^{c}
Protocatechuic acid	34,64 ± 0^{b}	45,82 ± 0^{a}	46,43 ± 0^{a}	44,78 ± 0,11^{a}	40,29 ± 2^{a}	ND^{c}
Catechin	10,08 ± 0^{c}	21,73 ± 8,63^{b}	39,93 ± 4,59^{a}	15,87 ± 5,65^{b}	13,46 ± 8,46^{b}	11,74 ± 0^{c}
Salicylic acid	39,57 ± 0^{c}	82,51 ± 8,41^{a}	89,62 ± 6,09^{a}	77,25 ± 5,69^{b}	57,77 ± 10,34^{b}	42,38 ± 38,53^{c}
Rutin	2,75 ± 0^{b}	ND^{c}	ND^{c}	0,18 ± 0^{c}	11,41 ± 0,05^{a}	1,93 ± 1^{b}
Hisperidin	40,91 ± 0^{c}	72,43 ± 66,4^{a}	82,24 ± 4,20^{a}	62,64 ±18,17^{b}	42,84 ± 37,91^{c}	39,98 ± 18,59^{c}
Epicatechin	44,71 ± 17,13^{c}	50,40 ± 8,33^{a}	64,86 ± 15,88^{a}	55,88 ± 6^{a}	22,92 ± 2,42^{b}	39,08 ± 1^{c}
Syringic acid	ND^{c}	50,55 ± 2,88^{a}	37,69 ± 0,10^{b}	23,15 ± 2,56^{b}	30,15 ± 17,35^{b}	28,23 ± 30,97^{b}
Vanillic acid	32,25 ± 0^{c}	44,26 ± 5,09^{c}	74,78 ± 93,47^{a}	59,46 ±2^{b}	59,83 ± 0^{b}	45,71 ± 0^{c}
p-Coumaric acid	3,26 ± 00^{c}	26,19 ± 8,64^{a}	27,99 ± 11,30^{a}	28,49 ±9,04^{a}	22,94 ± 5,76^{a}	10,90 ± 7,96^{b}

* ND—Not Detected. The presented values are Means ± SD, n = 3. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

4.2.2 Antioxidant activity

Analysis of antioxidant activity by 4 methods (Table 14) shows that during the first days of cultivation an increase in antioxidant potential is observed, which in the last days significantly decreases. The experiment shows that the results between the 9th and 12th days of cultivation are statistically indistinguishable for 3 of the methods – CUPRAC (day 9 - 55.19 ± 7.16 µM TE/g and day 12 - 50.6 ± 5.27 µM TE/g), FRAP (day 9 - 10.50 ± 0.45 µM TE/g and day 12 - 9.56 ± 0.48 µM TE/g) and DPPH (day 9 - 6.17 ± 0.6 µM TE/g and day 12 - 5.64 ± 0.41 µM TE/g).

The correlation analysis conducted between the accumulation of total polyphenols and DPPH (r=0.955), CUPRAC (r=0.850), FRAP (r=0.844), TEAC (r=0.744) showed the highest degree of correlation on day 9 of cultivation.

Таблица 14. Антиоксидантна активност на динамика на развитие на клетъчна суспензионна култура

	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21
Cu-(II)-reducing activity, µM TE/g dry biomass	46,60 ± 5,79^{b}	51,39 ± 6,71^{a}	55,19 ± 7,16^{a}	50,6 ± 5,27^{a}	58,65 ± 6,32^{a}	41,73 ± 5,49^{b}	37,65 ± 4,48^{c}
Fe-(III)-reducing activity, µM TE/g dry biomass	4,84 ± 1,69^{c}	11,89 ± 1,4^{a}	10,50 ± 0,45^{a}	9,56 ± 0,48^{a}	11,74 ± 0,77^{a}	8,07 ± 1,02^{b}	5,01 ± 0,49^{c}
DPPH-radical scavenging activity, µM TE/g dry biomass	4,08 ± 0,63^{b}	5,30 ± 0,9^{b}	6,17 ± 0,6^{a}	5,64 ± 0,41^{a}	7,02 ± 0,52^{a}	3,28 ± 0,56^{c}	2,08 ± 0,83^{c}
ABTS radical scavenging activity, µM TE/g dry biomass	9,40 ± 3,18^{c}	19,89 ± 4,01^{a}	18,86 ± 2,69^{a}	15,85 ± 3,73^{b}	27,91 ± 2,63^{a}	10,47 ± 2,63^{b}	9,31 ± 1,34^{c}
Total polyphenols, µM GAE/g dry biomass	2,37 ± 0,24^{b}	2,26 ± 0,69^{b}	7,80 ± 0,2^{a}	7,58 ± 0,15^{a}	7,9 ± 0,2^{a}	1,55 ± 0,31c	1,49 ± 0,54^{c}

* ND—Not Detected. The presented values are Means ± SD, n = 3. Means that do not share a letter are significantly different (p < 0.01, ANOVA with Tukey pairwise comparisons of means).

4.2.3 Ammonium, nitrate and phosphate ions

The study of ammonium, nitrate and phosphate ions (figures 13, 14 and 15) is an important indicator of the development of the culture during the specified days of cultivation. Their uptake from the nutrient medium is an important indicator of the growth and production of polyphenolic compounds. The graphs show that during the first days of cultivation, ammonium, nitrate and phosphate ions are most intensively absorbed, which is associated with the moment of production of the polyphenolic compounds from the table and antioxidant activity. It can be seen that by the 9th day

they are absorbed by the culture, i.e. their concentrations in the nutrient medium are decisive for the production of metabolites and the initial stage of this process.

4.3 Two-factor optimization of cell suspension culture

For the purposes of the present experiment, cell suspension culture was cultivated on WP medium, but with a different combination of sucrose concentration and medium salts for 9 days.

The following combinations were used:

1 – 15 g/L sucrose, ½ WP, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine

2 - 45 g/L sucrose, ½ WP, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine

3 - 15 g/L sucrose, 1.5 WP, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine

4 - 45 g/L sucrose, 1.5 WP, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine

5 - 30 g/L sucrose, WP, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine

4.3.1 Accumulated dry biomass and secondary metabolites

When cultivating cell suspension culture on nutrient media with different sucrose and salt content of the medium

From Figure 16 presenting the results for dry biomass accumulation, it can be clearly seen that the maximum yield of 52.02 ± 1.03 g/L is obtained when cultivating nutrient media enriched with 30 g/L sucrose, WP, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine. The lowest yield of 7.05 ± 0.02 g/L is obtained when cultivating nutrient media enriched with 15 g/L sucrose, ½ WP, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine.

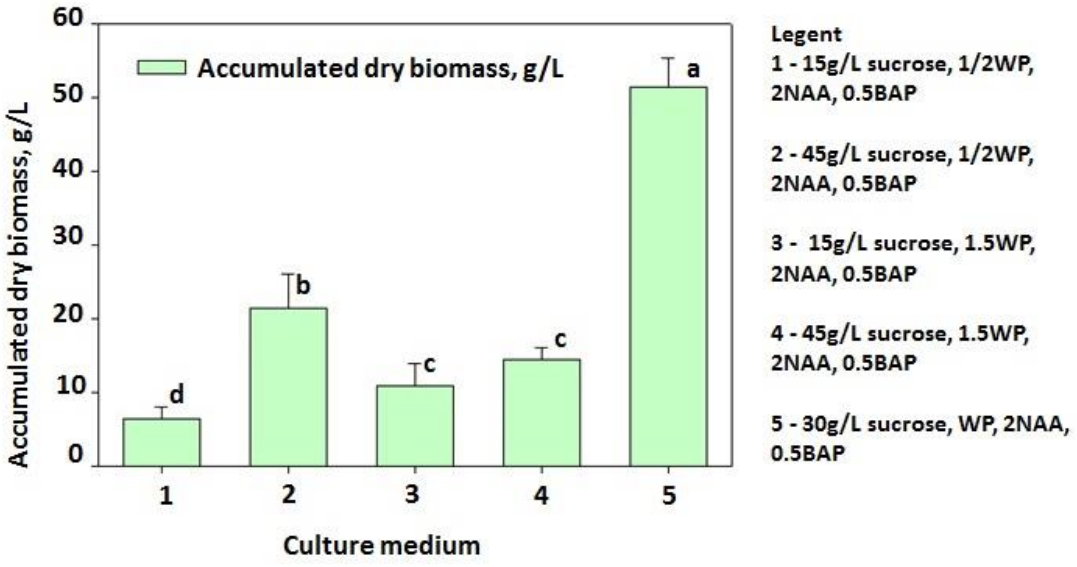


Figure 16. Tracking the accumulated dry biomass during cell suspension culture on nutrient media with different sucrose and salt content in the medium

The next important stage of the experiment is the monitoring of the accumulation of polyphenolic compounds from the suspension culture cultivated on the selected nutrient media.

The data from Table 15 show that chlorogenic acid is found in the highest concentration in nutrient medium number 5 (30g/L sucrose, WP, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine) 48.15 ± 2.78µg/g, and in the lowest concentration in nutrient medium number 1 (15g/L sucrose, 1/2WP, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine) 10.28 ± 1.27 µg/g, while in the cultures cultivated on the other nutrient media it is not found at all.

Table 15. Monitoring the accumulation of polyphenolic compounds from the suspension culture cultivated on the selected nutrient media.

	15g/L sucrose, 1/2WP, 2NAA, 0.5BAP	**45g/L sucrose, 1/2WP, 2NAA, 0.5BAP**	**15g/L 3 sucrose, 1.5WP, 2NAA, 0.5BAP**	**45g/L sucrose, 1.5WP, 2NAA, 0.5BAP**	**30g/L sucrose, WP, 2NAA, 0.5BAP**
Chlorogenic acid, µg/g	10,28 ± 1,27^{b}	ND^{c}	ND^{c}	ND^{c}	32,15 ± 2,78^{a}
Caffeic acid, µg/g	ND^{c}	ND^{c}	15,75 ± 0,83^{b}	21,73 ± 1,77^{a}	23,44 ± 0,99^{a}
Ferulic acid, µg/g	11,90 ± 2,34^{b}	9,97 ± 2,98^{b}	11,27 ± 2,16^{b}	12,24 ± 2,19^{b}	15,34 ± 1,95^{a}

Rosmarinic acid, µg/g	25,43 ± 1,38^{c}	34,31 ± 6,20^{b}	20,66 ± 2,32^{c}	39,13 ± 2,67^{b}	96,19 ± 0,76^{a}
Quercetin, µg/g	28,03 ± 2,98^{c}	75,07 ± 3,27^{b}	21,60 ± 17,34^{c}	71,30 ± 5,36^{b}	148,26 ± 4,97^{a}
Kaempferol, µg/g	32,46 ± 3,98^{b}	15,49 ± 4,24^{c}	7,82 ± 5,02^{c}	39,43 ± 4,00^{b}	60,37 ± 1,34^{a}
Gallic acid, µg/g	10,63 ± 4,74^{c}	8,96 ± 1,95^{c}	10,17 ± 4,31^{c}	13,22 ± 2,78^{b}	16,95 ± 5,51^{a}
Protocatechinic acid, µg/g	33,74 ± 4,43^{b,c}	46,48 ± 0,79^{b}	25,27 ± 7,25^{c}	41,32 ± 3,34^{b}	52,76 ± 12,91^{a}
Catechin, µg/g	ND^{b}	ND^{b}	30,14 ± 2,33^{a}	32,55 ± 7,23^{a}	34,99 ± 13,48^{a}
Salicylic acid, µg/g	32,34 ± 4,58^{b}	47,73 ± 3,44^{b}	13,06 ± 3,08^{c}	65,92 ± 4,01^{a}	72,23 ± 3,60^{a}
Rutin, µg/g	27,08 ± 0,63^{c}	26,76 ± 1,73^{c}	24,81 ± 8,27^{c}	35,62 ± 6,86^{b}	49,14 ± 7,48^{a}
Hisperidin, µg/g	24,99 ± 0^{c}	27,60 ± 1,07^{c}	43,55 ± 3,897^{b}	74,71 ± 4,76^{a}	50,36 ± 0,28^{b}
Epicatechin, µg/g	29,78 ± 0^{c}	47,10 ± 4,51^{b}	45,43 ± 5,48^{b}	48,97 ± 8,29^{a}	51,01 ± 3,86^{a}
Syringic acid, µg/g	6,03 ± 3,47^{c}	25,44 ± 6,53^{a}	8,92 ± 1,31^{c}	10,35 ± 0,3^{b}	10,65 ± 2,2^{b}
Vanillic acid, µg/g	95,24 ± 8,27^{b}	89,63 ± 2,39^{b}	45,90 ± 10,7^{c}	99,62 ± 8,48^{b}	298,20 ± 0,2^{a}
p-Comic acid, µg/g	8,85 ± 0,006^{c}	16,24 ± 1,87^{b}	11,46 ± 3,92^{c}	22,69 ± 0,03^{a}	15,31 ± 5,60^{b}

* ND—Not Detected. The presented values are Means ± SD, $n = 3$. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

4.3.2 Antioxidant activity

The analysis for antioxidant activity (Table 16) shows that the highest antioxidant potential is possessed by cultures cultivated on medium number 5 - 30g/L sucrose, WP, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine (Table 22).

The best potential for reducing copper ions is possessed by the culture cultivated on medium 5, or 45.48 ± 1.14 µM TE/g, and the lowest on medium 1 - 7.30 ±0.48 µM TE/g. Statistically indistinguishable are the data for the reduction of iron ions in nutrient medium 4 and 5, respectively 9.82 ± 1.26 µM TE/g and 11.58 ± 2.40 µM TE/g, as well as in terms of the potential for capturing ABTS radical, respectively 1.59 ± 0.41 µM TE/g and 2.20 ± 1.57 µM TE/g. With the highest potential against the DPPH radical is the culture cultivated on nutrient medium number 5 - 26.58 ± 2.14 µM TE/g and with the lowest nutrient medium 1, respectively 8.99 ± 1.89 µM TE/g.

The results for total polyphenol content show statistically indistinguishable results for the cultures cultivated on harmful medium 4 and 5, as was also observed from the results of the HPLC analysis, 1.81 ± 0.48 µM GAE/g and 2.01 ± 0.42 µM GAE/g, respectively.

Table 16. Monitoring the antioxidant activity of cell suspension culture cultivated on selected nutrient media.

	15g/L sucrose, 1/2WP, 2NAA, 0.5BAP	45g/L sucrose, 1/2WP, 2NAA, 0.5BAP	15g/L sucrose, 1.5WP, 2NAA, 0.5BAP	45g/L sucrose, 1.5WP, 2NAA, 0.5BAP	30g/L sucrose, WP, 2NAA, 0.5BAP
Cu-(II)-reducing activity, µM TE/g dry biomass	10,28 ± 1,27^{b}	ND^{c}	ND^{c}	ND^{c}	32,15 ± 2,78^{a}
Fe-(III)-reducing activity, µM TE/g dry biomass	ND^{c}	ND^{c}	15,75 ± 0,83^{b}	21,73 ± 1,77^{a}	23,44 ± 0,99^{a}
DPPH-radical scavenging activity, µM TE/g dry biomass	11,90 ± 2,34^{b}	9,97 ± 2,98^{b}	11,27 ± 2,16^{b}	12,24 ± 2,19^{b}	15,34 ± 1,95^{a}
ABTS radical scavenging activity, µM TE/g dry biomass	25,43 ± 1,38^{c}	34,31 ± 6,20^{b}	20,66 ± 2,32^{c}	39,13 ± 2,67^{b}	96,19 ± 0,76^{a}
Total polyphenols, µM GAE/g dry biomass	28,03 ± 2,98^{c}	75,07 ± 3,27^{b}	21,60 ± 17,34^{c}	71,30 ± 5,36^{b}	148,26 ± 4,97^{a}

* ND—Not Detected. The presented values are Means ± SD, n = 3. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

4.3.3 Optimization plot and equation

The optimization of the cell suspension culture was performed according to two main parameters: salt concentration and sucrose in the culture medium using a software product (MINITAB 10.0). The results of the analysis confirmed the data from the experiment for monitoring the polyphenol content and antioxidant activity, and the optimal medium for the development of the culture was selected as WP, enriched with 30g/L sucrose, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine (Figure 17).

5. Optimization of the germination culture cultivation regime. Dynamics. Optimization

5.1 Optimization of the germination culture cultivation regime.

In practice, scaling up the process for the production of active ingredients from callus and suspension cultures is laborious and fraught with a number of challenges. Therefore, a more optimal option for the propagation of in vitro culture and the production of secondary metabolites for various purposes in the cosmetics, medicine and food industry is the scaling up of the processes through the use of a bioreactor system. In this case, we have chosen to work with a bioreactor system with temporary stirring /type RITA/ (Fig.13, Fig.14, Fig.15).

In 2021, Agustine Christela Melviana and his team (Melviana A. C., et al. 2021) investigated the influence of different stirring periods on an in vitro culture of S. rebaudiana cultivated in bioreactors with temporary stirring and proved that different stirring and aeration regimes are a critical moment that significantly affects the growth of the culture. The experiment proved that the production of secondary metabolites is at a higher concentration with 15 min stirring every 6 hours than with 15 min stirring every 3 hours and, in addition, the rate of absorption of substances is directly affected by the different cultivation periods (Melviana A. C., et al. 2021).

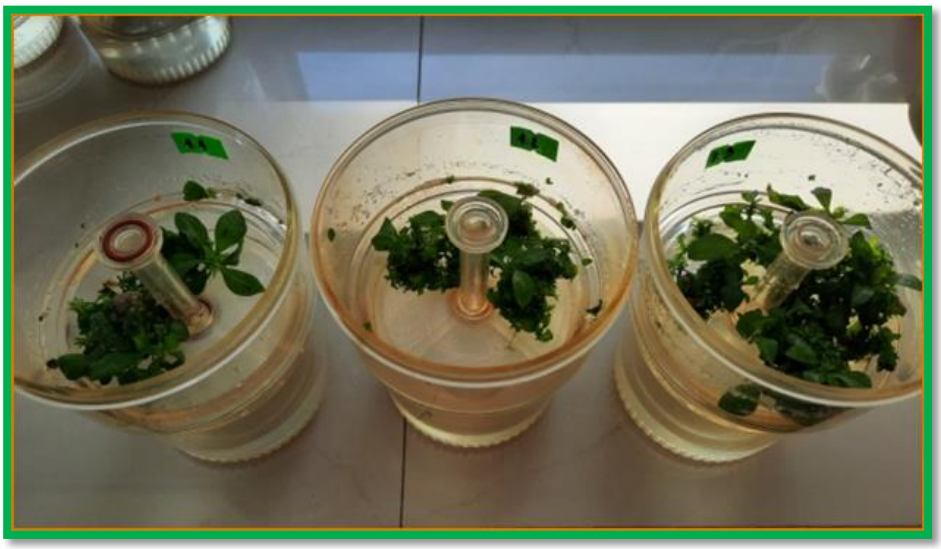


Figure 13. Mode 1 - 15 min aeration/4 hours rest

Figure 14. Mode 2 - 15 min aeration/8 hours rest

Figure 15. Mode 3 – 15 min aeration/12 hours rest

5.1.1 Accumulated dry biomass when choosing a cultivation mode

The presented figure 21 shows us that the largest amount of accumulated dry biomass was obtained when cultivating a mode with 15 min aeration and 12 hours of rest, respectively 32.02 ± 1.03 g/L and the least when cultivating 15 min aeration with 4 hours of rest 14.02 ± 2.13 g/L (Figure 16).

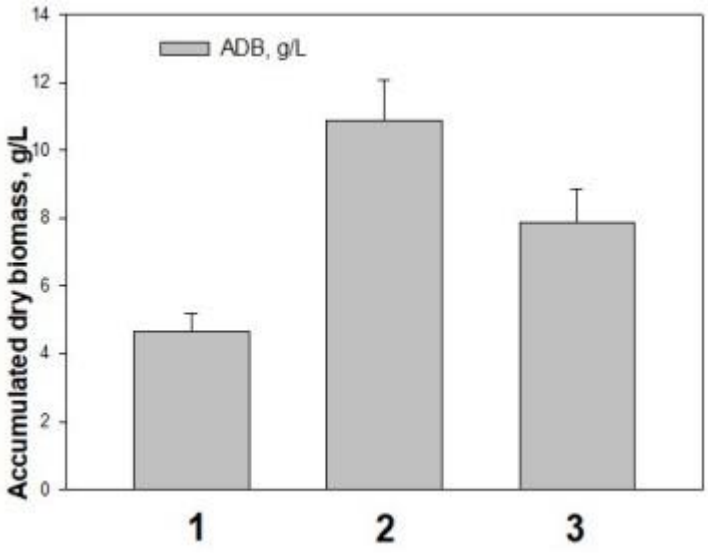


Figure 16. Monitoring dry biomass accumulation, 1 – Regime 1- 15 min immersion every 4 h 2 – Regime 2 - 15 min immersion every 8 h 3 – Regime 3 - 15 min immersion every 12 h

There is a lot of evidence in the literature on how the aeration regime affects biomass production and crop growth. For example, to increase the growth rate of Charybdis numidica, the immersion frequency was doubled from 5 min/24 h to 5 min/12 h. However, it turned out that the shoots became very sensitive to prolonged contact with the nutrient medium, resulting in completely hyperhydrated shoots (Wawrosch, C., 2005). The effect of a temporary agitation system with two, four and six immersions per day on the biomass production of Cymbopogon citratus was investigated. The maximum yield values of 66.2 g were obtained with four and six immersions per day treatments, while the dry biomass values (6.4 g) were higher with a frequency of four immersions per day (Quiala, E., 2006). Significant and distinct differences were recorded by Ahmadian and his team (Ahmadian, M., 2017) in vitro propagation of Dianthus caryophyllus using a temporary agitation system. Although increasing the immersion time induced the highest number of new shoots, the highest percentage of

hyperhydration was observed. Since hyperhydration is a notable sign of inefficiency of these systems, the most desirable is four immersions of 2 minutes/day.

5.1.2 Synthesis of secondary metabolites under different cultivation regimes

The results presented in Table 17 show that in the accumulation of the majority of polyphenolic compounds, there are no significant differences in their concentration under different cultivation regimes. Different cultivation regimes have an impact on some of the produced metabolites, for example, the highest concentration of rutin was reached in regime 3 (891.67 ± 122.29 µg/g) and the lowest in regime 2 (126.85 ±0 µg/g).

In 2015, a team of scientists (Ivanov. I., 2011) found that the biosynthesis of alkaloids from L. Aestivum is significantly affected by the cultivation regime of the sprout culture. In order to optimize the conditions for the production of secondary metabolites and achieve optimal biomass yield, not only the nutrient medium is important, but also the optimal aeration regime of the culture in the bioreactor.

Table 17. Tracking the accumulation of polyphenols during cultivation of different regimes

	4 hours rest, 15min aeration	8 hours rest, 15min aeration	12 часа покой, 15мин аерация(Режим 3)
Chlorogenic acid, µg/g	123,83±18,93*[a]	158,84±15,34[a]	164,82±32,36[a]
Caffeic acid, µg/g	23,13±5,9[a]	18,83±6,68[a]	26,90±6,80[a]
Ferulic acid, µg/g	103,06±10,97[a]	55,56±3,16[a]	39,47±4,51[a]
Salicylic acid, µg/g	313,62±38,17[a]	284,49±117,04[a]	327,86±118,12[a]
Rutin, µg/g	519,65±11,11[b]	126,85±0[c]	891,67±122,29[a]
Hisperidin, µg/g	126,72±0[b]	132,94±6,07[a]	130,90±10,55[a,]
Rosmarinic acid, µg/g	355,24±38,25[b]	558,48±2,69[a]	559,19±19,85[a]
Quercetin, µg/g	204,48±16,63[a]	306,46±77,25[a]	60,10±13,91[a]
Kaempferol, µg/g	22,84±2,28[a]	37,47±8,61[a]	11,09±2,71[a]

* ND—Not Detected. The presented values are Means ± SD, $n = 3$. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

5.1.3 Antioxidant activity

The results in Table 18 clearly show that the measurement of the antioxidant activity of methanol extracts from the seedling culture of jasmine gardenia, there are no major differences in the different cultivation regimes. However, when statistical analysis was performed, the correlation was highest in regime 3 (15 min. aeration/12 h rest) between the method for determining total polyphenols and the other methods and was respectively for CUPRAC (r = 0.816), FRAP (r = 0.823), DPPH (r = 0.754), ABTS (r = 0.743). While in mode 1 (15 min. aeration/4 h rest) CUPRAC (r = 0.516), FRAP (r = 0.497), DPPH (r = 0.456), ABTS (r = 0.323) and mode 2 (15 min. aeration/8 h rest) CUPRAC (r = 0.421), FRAP (r = 0.396), DPPH (r = 0.322), ABTS (r = 0.318) the correlation is low.

Based on the results obtained for dry biomass production, accumulation of polyphenolic compounds and antioxidant activity for the next part of the experiments, namely determining the dynamics of culture development and optimization of the nutrient medium, we choose to work with mode 3 (15 min. aeration and 12 h rest).

Table 18. Monitoring of antioxidant activity during cultivation of different regimes

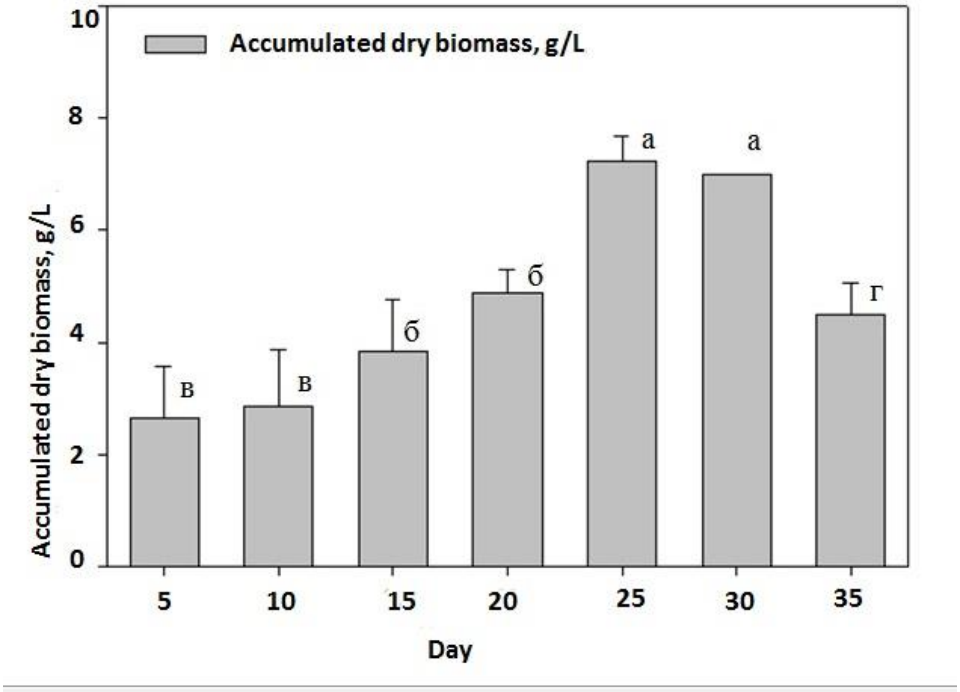
	4 hours rest, 15min aeration	**8 hours rest, 15min aeration**	**12 hours rest, 15min aeration**
Cu-(II)-reducing activity, µM TE/g dry biomass	39,66±5,8*[a]	37,60±5,71[a]	48,60±9,56[a]
Fe-(III)-reducing activity, µM TE/g dry biomass	14,10±0,5[a]	13,23±1,49[a]	14,14±0,56[a]
DPPH-radical scavenging activity, µM TE/g dry biomass	18,45±1,91[b]	21,32±1,14[b]	28,32±6,56[a]
ABTS radical scavenging activity, µM TE/g dry biomass	10,73±6,26[a]	12,47±1,7[a]	11,73±3,90[a]
Total polyphenols, µM GAE/g dry biomass	2,25±0,19[a]	2,28±0,57[a]	2,32±0,52[a]

* ND—Not Detected. The presented values are Means ± SD, $n = 3$. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

5.2 The dynamics of a seedling culture

5.2.1 Determination of the accumulation of dry biomass

An increase in the amount of accumulated dry biomass is observed during the first days, with the difference in yield between 5 and 10 days being statistically indistinguishable, and the maximum yield being reached on the 25th day, respectively the amount is 7.88 ± 0.22 g/L and on the 30th day where the yield is 7.79 ± 0.01 g/L. The statistical analysis conducted shows that the difference between the 25th and 30th day is insignificant. However, the values for the accumulation of dry biomass between the 30th and 35th day are statistically distinguishable, where the amount of produced biomass is 4.78 ± 0.85, or the production of dry biomass significantly decreases after the 30th day (Figure 17).



Фигура 27. Accumulated dry biomass, g/L

5.2.2 Analyzing changes in the absorption of ammonium, nitrate and phosphate ions

For the absorption of ammonium, nitrate and phosphate sources from the nutrient medium clearly demonstrate that during the first days of cultivation the sprouting

culture intensively absorbs nutrients, which also explains the increase in dry biomass production during these day**s.**

5.2.3 Secondary metabolite synthesis

The statistical analysis performed to determine the statistically distinguishable results of the yield of polyphenolic compounds shows that up to day 30 we observe an increase in the produced metabolites, after which their production sharply decreases. The concentrations of the metabolites on day 35 are close to those on the first days of cultivation and statistically significantly lower than those on day 30 (Table 19).

Таблица 19. Проследяване на промените в акумулирането на вторични метаболити

	Ден 5, µg/g	**Ден 10, µg/g**	**Ден 15, µg/g**	**Ден 20, µg/g**	**Ден 25, µg/g**	**Ден 30, µg/g**	**Ден 35, µg/g**
Chlorogenic acid	257,67 ± 48,52^{c}	442,29 ± 20,08^{b}	400,25 ± 10,26^{b}	379,22 ± 33,65^{b}	399,28 ± 12,65^{b}	482,65 ± 4,69^{a}	266,15 ± 7,04^{c}
Caffeic acid	94,54 ± 21,79^{b}	77,50 ± 55,47^{b}	123,71 ± 54,96^{a}	94,61 ± 6,71^{b}	95,81 ± 2,36^{b}	126,85 ± 59,89^{a}	36,91 ± 12,62^{c}
Ferric acid	62,84 ± 8,81^{b}	123,25 ± 55,30^{a}	103,40 ± 0,1^{a}	99,65 ± 30,93^{a}	67,42 ± 0,71^{b}	61,10 ± 2^{b}	122,55 ± 18,61^{a}
Rutin	126,60 ± 52,54^{c}	269,59 ± 20,39^{b}	361,15 ± 141,18^{b}	332, ± 15,47^{b}	222,19 ± 27,49^{b}	528,82 ± 191,00^{a}	154,29 ± 3,68^{c}
Rhoamaric acid	23,15 ±0^{c}	697,99 ± 302,08^{b}	1108,51 ± 174,05^{a}	1044,78 ± 8,69^{a}	612,35 ± 137,96^{b}	1182,64 ± 401,82^{a}	670,27 ± 169,01^{b}
Quercetin	232,99 ± 5,76^{b}	289,38 ± 61,43^{b}	276,57 ± 11,35^{b}	482,67 ± 23,52^{a}	330,81 ± 3,44^{a}	493,81 ± 26,8^{a}	109,43 ± 116,00^{c}
Kaempferol	ND^{d}	42,40 ± 2,3^{c}	66,13 ± 1,56^{b}	158,07 ± 14,01^{a}	116,14 ± 16,42^{a}	63,31 ± 8,22^{b}	144,68 ± 3,87^{a}
Salicylic acid	254,129 ± 37,87^{b}	263,95 ± 3,24^{b}	286,71 ± 20,72^{b}	516,80 ± 22,53^{b}	296,32 ± 20,22^{b}	660,99 ± 3^{a}	210,99 ± 15,73^{b}

Hisperidin	ND[c]	4,75 ± 1,54[c]	4,59 ± 0,69[c]	13,97 ± 11,02[b]	24,54 ± 3,9[b]	33,74 ± 21,81[a]	6,87 ± 0,7[c]

* ND—Not Detected. The presented values are Means ± SD, $n = 3$. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

5.2.4 Antioxidant activity

The antioxidant potential of the analyzed cultures shows that the results are statistically distinguishable between the 30th day and the first days of cultivation and those of the 35th day, when they are significantly lower. The correlation analysis conducted between the accumulation of total polyphenols and DPPH (r=0.905), CUPRAC (r=0.899), FRAP (r=0.856), TEAC (r=0.844) shows the highest degree of correlation at the 30th day of cultivation (Table 20).

Таблица 20. Проследяване на промените в антиоксидантната активност

	Day 5	**Day 10**	**Day 15**	**Day 20**	**Day 25**	**Day 30**	**Day 35**
Cu-(II)-reducing activity, µM TE/g dry biomass	21,82 ± 3,29[г]	55,26 ± 3,88[б]	48,98 ± 4,08[в]	69,41 ± 11,22[а]	33,51 ± 7,50[г]	41,91 ± 4,73[в]	45,81 ± 0,84[в]
Fe-(III)-reducing activity, µM TE/g dry biomass	15,94 ± 4,69[в]	23,49 ± 4,85[б]	25,8 ± 4,11[б]	27,93 ± 2,77[б]	37,69 ± 2,87[а]	38,59 ± 2,42[а]	21,25 ± 0,77[б]
DPPH-radical scavenging activity, µM TE/g dry biomass	19,85 ± 4,75[в]	17,21 ± 1,9[в]	23,06 ± 2,86[б]	26,47 ± 3,59[а]	25,97 ± 3,24[б]	30,31 ± 3,33[а]	16,27 ± 1,08[в]
ABTS radical scavenging activity, µM TE/g dry biomass	5,60 ± 2,69[в]	12,51 ± 0,46[б]	15,85 ± 1,14[б]	17,93 ± 0,79[а]	15,62 ± 0,79[б]	14,37 ± 1,54[б]	10,51 ± 0,72[в]

* ND—Not Detected. The presented values are Means ± SD, $n = 3$. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

5.3 Two-factor optimization

For the purpose of the present experiment, a cell suspension culture was cultivated on WP medium, but with a different combination of sugar and salt concentration of the medium for 30 days.

The content of the nutrient solution used in a temporary stirring system plays a key role in plant growth. It should also be noted that in some cases, liquid culture in a temporary stirring system can contribute to the use of a smaller number of mineral salts compared to conventional culture in a semi-solid medium. The use of liquid medium, in addition to reducing production costs by eliminating the gelling agent (Jova, M.C.. 2008) contributes to a more efficient use of the culture medium, since the explants are in direct contact with the liquid medium during the cultivation period and can absorb nutrients more efficiently (Watt, P., 2012).

The following combinations were used

1 – 15 g/L sucrose, ½ MS, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine

2 - 45 g/L sucrose, ½ MS, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine

3 - 15 g/L sucrose, 1.5 MS, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine

4 - 45 g/L sucrose, 1.5 MS, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine

5 - 30 g/L sucrose, MS, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine

5.3.1 Accumulation of dry biomass and synthesis of secondary metabolites

The maximum yield of dry biomass was achieved when cultivating on a nutrient medium of 30 g/L sucrose, MS, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine, respectively 17.05 ± 1.22 g/L dry biomass, and the lowest yield

was when cultivating on nutrient medium number 5 (15 g/L sucrose, 1.5 MS, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine) 7.55 ± 0.69 g/L dry biomass

The effect of the components present in the liquid culture medium is of great importance for the development of the culture in the system with temporary agitation, as demonstrated by Benelli and De Carlo (Benelli, C., 2018) for the micropropagation of olive trees using PlantForm with a cycle of 8 min/16 h.

Some authors have observed the effect of different concentrations of sucrose in the culture substrate on the biomass production. In particular, in the plant cultures Curcuma zedoaria, Zingiber zerumbet, Gynura procumbens and Bletilla striata, it has been proven that a higher concentration of sugar improves the efficiency of the explants (Zhang, B., 2018; Stanly, C., 2010; Manuhara, Y.S.W.p 2017).

The analysis carried out for the polyphenol content clearly shows the statistical differences when cultivating on nutrient media with different concentrations of sugar and salts (Table 21). It is noted that the highest yield of chlorogenic acid, rutin, quercetin, hesperidin and salicylic acid was found when cultivating on nutrient medium number 5 (30 g/L sucrose, MS, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine) or respectively 645.41 ± 56.66 µg/g, 19.86 ± 0.05 µg/g, 22.44 ± 3.37 µg/g, 252.62 ± 11.87 µg/g, 450 ± 15.97 µg/g. The lowest and statistically distinguishable from the cultivation on the other nutrient media were the metabolite concentrations in nutrient medium number 1 (15 g/L sucrose, ½ MS, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine).

Table 21. Accumulation of polyphenolic compounds

	15g/L sucrose, 1/2MS, 4BAP	**45g/L sucrose, 1/2MS, 4BAP**	**15g/L sucrose, 1.5MS, 4BAP**	**45g/L sucrose, 1.5MS, 4BAP**	**30g/L sucrose, MS, 4BAP**
Chlorogenic acid, µg/g	ND^{d}	28,30 ± 0,05^{c}	12,13 ± 1,49^{c}	288,78 ± 41,18^{b}	645,41 ± 56,66^{a}
Caffeic acid, µg/g	26,16 ± 1,24^{c}	46,34 ± 5,22^{b}	14,40 ± 2,82^{c}	22,41 ± 0,2^{c}	67,49 ± 18,76^{a}

Ferulic acid, µg/g	5,93 ± 0,28[a]	8,01 ± 2,25[a]	8,70 ± 1,45[a]	8,95 ± 0,64[a]	10,89 ± 0,22[a]
Rutin, µg/g	4,84 ± 0,9[b]	ND[c]	2,18 ± 0,19[b]	8,86 ± 0,24[b]	19,86 ± 0,05[a]
Rosmarinic acid, µg/g	188,77 ± 8,07[c]	263,03 ± 13,52[b]	360,39 ± 7,3[a]	314,74 ± 12,89[a]	365,05 ± 29,03[a]
Quercetin, µg/g	33,90 ± 0,8[a]	5,25 ± 0,09[c]	1,86 ± 0,19[c]	14,39 ± 0,09[b]	22,44 ± 3,37[a]
Kaempferol, µg/g	1,44 ± 0,006[c]	24,41490912[b]	33,25 ± 5,87[a]	33,15 ± 0,88[a]	29,78 ± 0,74[a]
Salicylic acid, µg/g	48,29 ± 5,57[c]	114,81 ± 13,89[b]	90,12 ± 4,72[b]	83,88 ± 9,81[b]	252,62 ± 11,87[a]
Hisperidin, µg/g	6,20 ± 0,9[c]	24,78 ± 2,72[b]	33,12 ± 7,65[b]	18,21 ± 4,13[c]	450,40 ± 15,97[a]

* ND—Not Detected. The presented values are Means ± SD, $n = 3$. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

Various examples are found in the literature on how modified culture media with fewer or more of the relevant components can influence the accumulation of phenolic compounds. For example, the content of total phenolic compounds, as well as the phenylethanoid glycosides verbascoside and isoverbascoside, increased in shoots of Castilleja tenuiflora exposed to modified culture medium B5 containing lower amounts of nitrogen, using a system with temporary agitation at 5 min/24 h (Medina-Pérez, V., 2015).

Also, the combination of an elicitor (Chitoplant®) and a two-vessel bioreactor with a temporary stirring system, as a reliable alternative for genetic modification, increases the production of cardenolides 2.2-fold in Digitalis lanata shoots (Pérez-Alonso, N., 2012) and significantly improves the galantamine content in the leaves and bulbs of Leucojum aestivum (Schumann, A., 2013) and stimulates the production of thapsigargin and nortrilobolide in vitro shoots of Thapsia garganica (López, C.Q., 2018). Furthermore, challenge induced the production and accumulation of phenylpropanoid metabolites in both the accumulated biomass and the culture medium of Aristotelia chilensis (Trentini, G.E., 2021)

Since the concentration and type of components are often considered to be a decisive factor for the accumulation of secondary metabolites, Langhansova and her team (Langhansova, L.. 2012) found that a medium containing benzo[b]selenenyl acetic acid and kinetin was most suitable for large-scale saponin production in Panax ginseng cultivated in a RITA® bioreactor system with temporary agitation.

5.3.2 Antioxidant activity

Statistical analysis clearly shows the highest antioxidant activity in cultures cultivated on nutrient medium number 5 - 15 g/L sucrose, ½ MS, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine (Table 22).

Table 22. Monitoring changes in antioxidant activity

	15g/L sucrose, 1/2MS, 4BAP	**45g/L sucrose, 1/2MS, 4BAP**	**15g/L sucrose, 1.5MS, 4BAP**	**45g/L sucrose, 1.5MS 4BAP**	**30g/L sucrose, MS, 4BAP**
Cu-(II)-reducing activity, µM TE/g dry biomass	27.50± 8.02^{c}	44.67 ± 3.14^{c}	34.99± 3.50^{c}	55.05± 2.33^{b}	48.72± 4.38^{a}
Fe-(III)-reducing activity, µM TE/g dry biomass	15.41± 1.52^{b}	17.17± 2.51^{b}	19.90± 1.47^{b}	22.30± 0.95^{a}	26.00± 3.85^{a}
DPPH-radical scavenging activity, µM TE/g dry biomass	12.30 ±1.11^{c}	20.22± 1.31^{b}	19.10 ±1.59^{b}	25.27± 1.59^{b}	25.01± 2.27^{a}
ABTS radical scavenging activity, µM TE/g dry biomass	2.73± 0.4^{c}	5.71± 0.56^{a}	4.24± 0.99^{b}	5.55± 0.98^{b}	4.69± 0.89^{b}

* ND—Not Detected. The presented values are Means ± SD, $n = 3$. Means that do not share a letter are significantly different ($p < 0.01$, ANOVA with Tukey pairwise comparisons of means).

5.3.3 Optimization plot and equation

The optimization of the cell suspension culture was performed according to two main parameters - salt concentration and sucrose in the nutrient medium, using a software product (MINITAB 10.0). The result of the analysis confirmed the data from the experiment for monitoring the polyphenol content and antioxidant activity, and the optimal medium for the development of the culture was selected as MS, enriched with 30g/L sugar, 2.0 mg/L 1-naphthylacetic acid and 0.5 mg/L 6-benzylaminopurine.

6. Monitoring genetic stability

The results of the molecular study show that the developed markers are adequate and suitable for the purposes of the dissertation, and can be used to monitor the genetic changes occurring in in vitro cultures of gardenia.

There is not enough available data in the literature regarding the study of the relationship between the duration of cultivation of an in vitro culture obtained from jasmine gardenia, changes in the composition of the culture medium and somoclonal variability.

Assumptions are made to what extent the conditions of in vitro cultivation affect genetic stability, and how this affects the variability in the number and structure of chromosomes (Moradi, Z., et al., 2017).

The longer a culture is maintained in vitro, the greater the possibility of somaclonal variation (Kuznetsova et al. 2006; Gao et al. 2010; Farahani et al. 2011; Jevremović et al. 2012; Sun et al. 2013).

Furthermore, tissue culture propagation techniques during micropropagation can affect its genetic stability.

Clarindo et al. (2012) concluded that it is necessary to subculture coffee aggregate cell suspensions for no more than 4 months, as ploidy instability has been observed during long-term in vitro cultivation.

Not only the number of subcultures, but also the duration also contribute to increasing the rate of somaclonal variation, especially in cell suspensions and callus cultures (Bairu et al. 2006; Sun et al. 2013). Studies have shown that somaclonal variation is more evident in plants regenerated from long-term in vitro cultures (Etienne and Bertrand 2003).

The data show (Table 23 and Figure 18) that all used SSRs have more than three alleles and can be used to assess the occurring genetic changes when monitoring the dynamics of the development of the cell suspension culture. The highest content of polymorphic information is given by the use of markers GJ04, GJ08, GJ09, GJ17, GJ03 respectively PIC=0.914, PIC=0.910, PIC=0.911, PIC=0.901, PIC=0.947. The number of detected Na alleles varies from 2 to 22 with an average of 13 ± 1.73, and the number of effective Ne alleles varies from 1 to 14 with an average of 8.16 ± 0.70, as the indicated values confirm the genetic variations that occurred during the cultivation of the cell suspension culture. This statement is confirmed once again by the values of expected and observed heterozygosity, respectively He = 0.98 ± 0.000 and Ho = 1 ± 0.

Table 23. Genetic stability during prolonged cultivation of cell suspension culture

SSR Locus	Na	Ne	Ho	He	PIC
eGJ010	2	1	1	1	0,375
eGJ118	0	0	0	0	0
eGJ144	0	0	0	0	0
GJ04	15	9	1	0,974	0,914
GJ08	12	6	1	1	0,91
GJ09	15	12	1	0,957	0,911
GJ10	0	0	0	0	0
GJ16	9	6	1	0,955	0,862
GJ17	12	7	1	0,978	0,901
GJ02	0	0	0	0	0
GJ03	22	14	1	0,984	0,947
Average	**13**	**8,166667**	**1**	**0,982167**	**0,826333**
SD	**1,732051**	**0,707107**	**0**	**0,00021**	**0,002082**

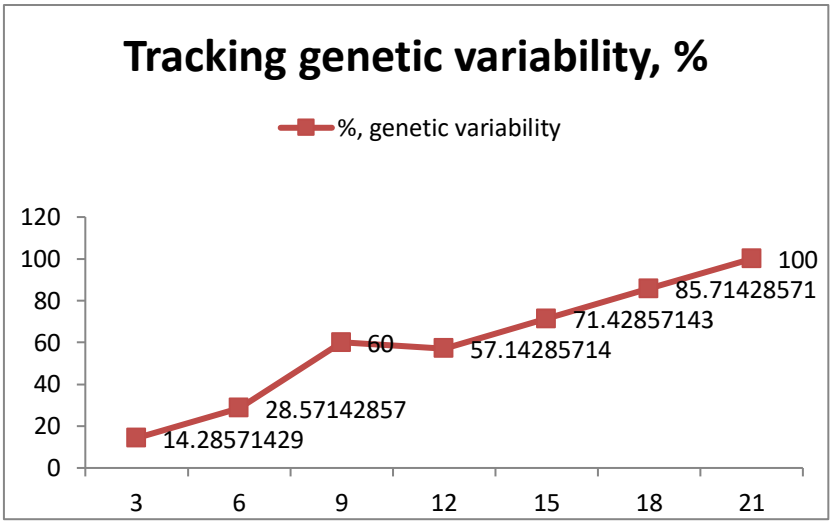


Figure 18. Tracking genetic variability

6.2 Genetic stability when changing the concentration of the components of the nutrient medium of cell suspension culture

The data show (Table 24) that of all the SSRs used, only 2 of them, namely GJ08 and GJ03, can be used to assess the genetic changes occurring during optimization of the development of cell suspension culture. The highest content of polymorphic information is given by the use of GJ08, respectively PIC=0.926, and GJ03 PIC=0.891.

Table 24. Genetic stability when changing the concentration of the components of the nutrient medium of cell suspension culture

SSR Locus	Na	Ne	Ho	He	PIC
eGJ010	0	0	0	0	0
eGJ118	0	0	0	0	0
eGJ144	0	0	0	0	0
GJ04	0	0	0	0	0
GJ08	17	10	1	0,979	0,926
GJ09	0	0	0	0	0
GJ10	0	0	0	0	0
GJ16	0	0	0	0	0
GJ17	0	0	0	0	0
GJ02	0	0	0	0	0
GJ03	11	12	1	0,938	0,891

Average	**14**	**11**	**1**	**0,9585**	**0,9085**
SD	**4,242641**	**1,414214**	**0**	**0,028991**	**0,024749**

6.3 Genetic variability in long-term cultivation of sprout culture

The number of detected Na alleles varied from 4 to 22 with an average of 16 ± 2.31, and the number of effective Ne alleles varied from 2 to 16 with an average of 10.66 ± 2.30, as the indicated values confirm the genetic variations that occurred during the cultivation of sprout culture. This statement is confirmed once again by the values of expected and observed heterozygosity, respectively He = 0.97 ± 0.010 and Ho = 0.91 ± 0.02 (Table 25)

Table 25. Genetic stability in long-term cultivation of shoot culture

SSR Locus	Na	Ne	Ho	He	PIC
eGJ010	0	0	0	0	0
eGJ118	0	0	0	0	0
eGJ144	4	2	1	1	0,703
GJ04	4	2	1	1	0,703
GJ08	15	10	1	0,958	0,904
GJ09	11	6	1	0,985	0,895
GJ10	0	0	0	0	0
GJ16	11	6	1	0,985	0,895
GJ17	4	2	1	1	0,703
GJ02	4	2	1	1	0,703
GJ03	22	16	1	0,978	0,945
Average	**16**	**10,66667**	**1**	**0,973667**	**0,914667**
SD	**2,309401**	**2,309401**	**0**	**0,012767**	**0,02388**

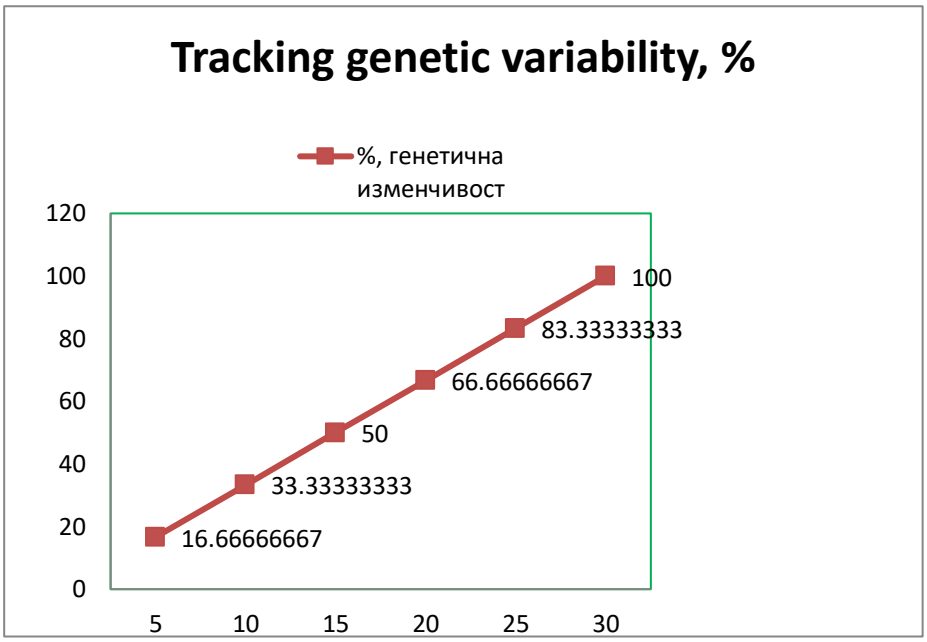


Figure 19. Tracking genetic variability

6.4 Genetic stability when changing the concentration of the components of the nutrient medium of a sprouting culture

The highest content of polymorphic information is given by the use of GJ08, respectively PIC=0.931, and GJ03 PIC=0.942. The number of detected Na alleles varies from 17 to 20 with an average of 18.5 ± 2.12, and the number of effective Ne alleles varies from 10 to 12 with an average of 11 ± 1.41, as the indicated values confirm the genetic variations that occurred during the cultivation of the cell suspension culture (Table 26).

This statement is confirmed once again by the values of expected and observed heterozygosity, respectively He = 0.98 ± 0.001 and Ho = 0.93 ± 0.007.

Accordingly, changing the nutrient medium in both suspension cultures and sprouting cultures has minimal impact on genetic stability.

Table 26. Genetic stability with changes in the concentration of the components of the nutrient medium of a sprouting culture

SSR Locus	Na	Ne	Ho	He	PIC
eGJ010	0	0	0	0	0
eGJ118	0	0	0	0	0
eGJ144	0	0	0	0	0
GJ04	0	0	0	0	0
GJ08	17	10	1	0,984	0,931
GJ09	0	0	0	0	0
GJ10	0	0	0	0	0
GJ16	0	0	0	0	0
GJ17	0	0	0	0	0
GJ02	0	0	0	0	0
GJ03	20	12	1	0,986	0,942
Average	**18,5**	**11**	**1**	**0,985**	**0,9365**
SD	**2,12132**	**1,414214**	**0**	**0,001414**	**0,007778**

It is seen that the cultivation of plant tissues in vitro leads to an increase in genetic variability. As can be seen from the results obtained, the level of genetic variability of cultured plant cells depends on the duration of their cultivation in vitro.

Young cell cultures in the first months of cultivation are characterized by an increase in heterogeneity (Armstrong, C.L. and Phillips, R.L 1988; Fras, A. and Maluszynska, J., 2003).

As noted in the analyses using SSR markers, the greatest changes in genetic stability in suspension and seedling cultures of jasmine gardenia are observed when following the duration of cultivation of the cultures. In some cultures, long-term cultured cells are usually characterized by stabilization of the genome, accompanied by maintenance of a constant level of ploidy and a decrease in the mutation rate (Cote, F.X., 2001; Kuznetsova, O.I., et al., 2006). One explanation is that long-term cultivation is the result of a long series of mitoses, during which multiple mutations occur spontaneously and uncontrolledly, and the greater the number of subcultures, the more these mutations can affect a larger amount of genetic material (Nwauzoma, A.B.,

2013). The effect of long-term in vitro cultivation on A. thaliana has not led to genome stability in studies conducted to date.

Accordingly, a clear conclusion for all types of in vitro cultures or plants, whether self-clonal variation changes depending on the cultivation period, cannot be made, because each plant has a different character in terms of genetic stability cultivated in different in vitro systems for different periods of time.

IV. CONCLUSIONS

1. The dynamics of growth and development of the studied in vitro cultures have been established and a nutrient medium has been optimized for the cultivation of differentiated (germinated) and dedifferentiated (cell suspension) in vitro cultures of Gardenia jasminoides Ellis

2. The germinated culture accumulates phenolic compounds in concentrations greater than or close to those of the parent plant, while in the callus and suspension culture the concentrations of phenolic compounds are lower. The antioxidant potential of the undifferentiated in vitro cultures (callus and cell suspension) is lower than that of the differentiated culture and gardenia leaves.

3. It has been established that genetic variability is most strongly dependent on the age of the culture and occurs with the highest intensity in the exponential phase of development. Changes in the composition of the nutrient medium indicate a minimal impact on changes in the genetic stability of the culture.

4. Depending on the degree of differentiation of the culture, studies have shown that the cell suspension has the highest genetic stability and proximity to that of the parent plant. The highest genetic variability is observed in the differentiated seedling culture.

5. Regarding primary metabolites, the genetic similarity between the plant and the cell culture is the highest, and in the biosynthesis of phenolic compounds, the differentiated seedling culture produces more phenolic compounds and even surpasses the parent plant.

7. It has been established that the differentiated seedling culture has the highest potential for developing a biotechnological method for the production of phenolic compounds from G. jasminoides Ellis with high

antioxidant potential. Due to the high risk of genetic variability, it is most rational to maintain the culture by cryopreservation or subculturing for shorter periods.

V. CONTRIBUTIONS

1. Differentiated (germination) and dedifferentiated (callus and cell suspension) in vitro cultures of Gardenia jasminoides Ellis were obtained

2. The differences in the metabolic profile and antioxidant activity of the obtained in vitro systems and Gardenia jasminoides Ellis were studied for the first time.

3. The genetic stability of a differentiated and dedifferentiated culture was studied for the first time during the study of the dynamics of development as well as during the change of the main components of the nutrient medium.

4. The genetic stability of differentiated and dedifferentiated in vitro systems was studied for the first time compared to that of the parent plant.

5. The relationships between changes in the metabolic profile and genetic variability during the cultivation of differentiated and dedifferentiated in vitro cultures of gardenia were established for the first time.

VI. PUBLICATIONS ON THE TOPIC OF THE DISSERTATION

1. Krasteva Gergana, Vasil Georgiev, and Atanas Pavlov; Recent applications of plant cell culture technology in cosmetics and foods; *Engineering in Life Sciences* 21, no. 3-4 (2021): 68-76. (**IF 2020 – 2.678**), **Q2**

2. Krasteva, Gergana, et al. "Metabolite Profiling of Gardenia jasminoides Ellis In Vitro Cultures with Different Levels of Differentiation." *Molecules* 27.24 (2022): 8906. **(IF 2022 – 4.600)**

3 Krasteva, Gergana. "Effect of basal medium on growth and polyphenols accumulation by Gardenia jasminoides Ellis cell suspension." *BIO Web of Conferences*. Vol. 45. EDP Sciences, 2022.

4 Krasteva, Gergana. "Cultivation of Gardenia jasminoides shoots in temporary immersion systems and optimization of the immersion periods." *BIO Web of Conferences*. Vol. 58. EDP Sciences, 2023.

Citations of publications related to the dissertation work /without self-citations/ - 136

PRESENTATIONS AT SCIENTIFIC EVENTS IN THE COUNTRY AND ABROAD

1. Presentation at a scientific event abroad – European federation of biotechnology 2021

2. Presentation at a scientific event in the country –

68TH SCIENTIFIC CONFERENCE WITH INTERNATIONAL PARTICIPATION – "FOOD SCIENCE, TECHNOLOGY AND TECHNOLOGY - 2021"

69TH SCIENTIFIC CONFERENCE WITH INTERNATIONAL PARTICIPATION – "FOOD SCIENCE, TECHNOLOGY AND TECHNOLOGY - 2022"