

Cultivation of *Gardenia jasminoides* shoots in temporary immersion systems and optimization of the immersion periods

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Abstract. In the present study, the effect of different immersion regimes on the growth and secondary metabolites production by shoot cultures of *Gardenia jasminoides* Ellis, cultivated in temporary immersion systems (TIS) was investigated. The cultivation was performed for 30 days on MS medium supplemented with 4.0 mg/L BAP. The regimes of cultivation included 15 min. immersion every 4, 8 and 12 hours. The highest amount of accumulated dry biomass (ADB = 10.86 ± 0.02 g/L) was reached at 15 min. immersion at every 8 hours, whereas the lowest (ADB = 4.65 ± 0.26 g/L) was recorded when 15 min. immersion on every 4 hours was used. HPLC analyses showed that the higher polyphenol content was achieved when shoots were cultivated by using 15 min. immersion on every 12 hours. At this conditions, the shoots produced maximal amounts of chlorogenic acid ($164.82 \pm 32.36 \mu\text{g/g DW}$), caffeic acid ($26.90 \pm 6.80 \mu\text{g/g DW}$), rutin ($891.67 \pm 122.29 \mu\text{g/g DW}$) and rosmarinic acid ($559.19 \pm 19.85 \mu\text{g/g DW}$). The observed antioxidant activity (DPPH, ABTS, CUPRAC and FRAP) was following the accumulation of total phenolic. The study demonstrates that TIS are effective for cultivation of *Gardenia jasminoides* shoots.

1 Introduction

Gardenia jasminoides Ellis has a rich number of phytochemicals and the extract from the plant are often used like natural colorant in traditional Chinese medicine for different diseases [1]. The last studies for the effect of *Gardenia jasminoides* extract on skin showed that it had an excellent effect on the treatment of atopic dermatitis. That made a *Gardenia jasminoides* Ellis very interesting for cosmetics products [2]. This is some of the reason that this plant is very interesting for food, chemical, textile, medicine and cosmetics industry. The information for optimization the process of production the secondary metabolites from *Gardenia jasminoides* shoots cultures are scarce. In recent years, the use of in vitro technologies to obtain commercial products and the production of various biologically active substances become a profitable industry [3]. To obtain a better yield with good growth and metabolic characteristics, cultivation in bioreactors with liquid nutrient medium is widely used [4]. Bioreactors are specially developed for highly sensitive plant crops. They allow cultivation under controlled conditions tailored to the requirements of the cell culture [4]. The control is fully computerized. Different bioreactor systems were developed for the needs of different types of cultivation – hybrid bioreactors, gas-phase bioreactors, temporary immersion systems (TIS), liquid-phase bioreactors. In recent years TIS were widely used for cultivation of plant shoots. This type of bioreactor is associated with cultivation under a certain

aeration regime. The period of air expose is several hours and the period of the immersion is a few minutes [4]. Temporally immersion systems protect the plants from the influence of mechanical stress. Typically, bioreactors are made of glass or plastic, which allows efficient light access. The use of this type of bioreactor cultivation is preferable in the production of secondary metabolites from differentiated cell cultures in the laboratory and initial scaling up of their production [5, 6, 7, 8, 9]. This bioreactor is also used to study the different pigment formation in some plants [10].

TIS is an extremely convenient and modernized approach to the production of secondary metabolites, which allows adaptation in larger scale production for the needs of the cosmetics and food industry. Here we report analysis and optimization of the immersion periods related to cultivation of *Gardenia jasminoides* Ellis shoots in TIS.

2 Materials and methods

2.1 Plant material

The initiation of shoot culture used in this study is described in Krasteva [11] and [12].

2.2 Cultivation conditions

Gardenia jasminoides shoots were grown in basal MS

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(Murashiga and Skoog) media supplemented with 30g/L sugar and 4 mg/L BAP for a period of 30 d. The shoot cultures were cultivated in temporary immersion systems (TIS) under different immersion regimes at 24°C. The studied regimes included 15 min. immersion every 4, 8 and 12 h, respectively. Following cultivation, the biomass was collected and the accumulated dry biomass (ADB) and the growth index dry weight (GIDW) were calculated.

2.3 Monitoring of growth

The assay was performed as described by Krasteva [12].

The dry content of the biomass was determined using a direct method where approximately 1 g of the sample was incubated in an oven at $90 \pm 2^\circ\text{C}$ until a constant weight was obtained. The samples were removed from the oven, tempered in a desiccator and weighed. The results were expressed as a percentage of dry matter.

2.4 Preparation of extracts

Biomass from 30-day old *G. jasminoides* shoot cultures were dried and milled to a fine powder by using laboratory homogenizer, 0.5 g of the dried biomass was used for triplicate extraction (3×10 mL methanol) in an ultrasonic bath for 15 min. at room temperature. After extraction methanol was evaporated under vacuum at 50°C and dry residue was dissolved in pure methanol (2 ml) and was used for analysis of polyphenols and antioxidant activity.

2.5 HPLC analysis of phenolics

Phenolics were analyzed on a SUPELCO Analytical Discovery HS C18 column (25 cm x 4.6 mm, 5 μm) (Sigma, Germany) using Waters 1525 binary HPLC pump and Waters 2487 UV-VIS detector. The injected sample was 20 μL . The HPLC conditions were according to Kristeva [12].

2.6 Antioxidant activity analyzes

2.6.1 DPPH scavenging assay

The assay was performed described by Ivanov et al. [13] with some modifications.

Two hundred and eighty microliters of 0.04 mg/mL freshly prepared solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma, Germany) in methanol were mixed with 20 μL sample and the light absorption was measured 515 nm after 15 min incubation at 37°C in darkness on Multiscan FC multiplate reader (ThermoScientific, USA). The results were expressed as μM equivalent of Trolox (TE) per g dry weight (DW), according to a calibration curve, built in range of 0.05 – 0.5 mM Trolox (Sigma, Germany).

2.6.2 ABTS scavenging assay

The assay was performed according as described by Ivanov et al. [13] with some modifications. ABTS (2, 20-

azino-bis (3-ethylbenzothiazoline6-sulfonic acid) cation radical was generated by mixing aliquot parts of 3.84 mg/mL of ABTS (Sigma) in distilled water and 2.45 mM potassium persulfate (Merck) in distilled water. Two hundred and eighty microliters of ABTS cation radical were mixed with 20 μL sample and the light absorbance was measured at 734 nm after 15 min incubation at 37°C in darkness on a Multiscan FC multiplate reader (ThermoScientific, USA). The results were expressed as μg equivalents of Trolox (TE) per g dry weight (DW), according to a calibration curve, built in the range of 0.05 - 0.5 μg Trolox (Sigma, Germany).

2.6.3 FRAP Assay

The ferric reducing antioxidant power (FRAP) assay was performed as described by Ivanov et al. [13] with some modifications. FRAP reagent was prepared by mixing 10 parts sodium acetate buffer (pH 3.6), 1 part of 3.1 M 2,4,6-tris (2-pyridyl)-s triazine (TPTZ) in 40 mM HCL and one part of 20mM FeCl_3 in H_2O . Two hundred and eighty microliters of freshly prepared FRAP reagent were mixed with 20 μL sample and the light absorbance was measured at 595nm after 10 min incubation at 37 °C in darkness on a Multiscan FC multiplate reader (ThermoScientific, USA). The results were expressed as μM equivalents of Galic acid (GAE) per g dry weight (DW), according to a calibration curve, built in the range of 0.05 – 0.5 mM Trolox (Sigma, Germany).

2.6.4 Cupric reducing antioxidant capacity (CUPRAC) assay

The Cupric Reducing Antioxidant Capacity (CUPRAC) assay was performed as described by Ivanov et al. [13] with some modifications on the light absorbance, quantity of reagents and analyzed sample.

The 20 μL sample, 70 μL 10 mmol CuCl_2 , 70 μL 0.1 M ammonium acetate buffer (pH 7.0), 70 μL 7.5 mM Neocuproine and 70 μL distilled water were used.

The light absorbance was measured at 450 nm after 30 min incubation at 37°C in darkness on Multiscan FC multiplate reader (ThermoScientific, USA). The results were expressed as μg Trolox equivalents (TE) per g dry weight (DW), according to a calibration curve, built in the range of 0.05 - 0.5 mM Trolox (Sigma, Germany).

2.6.5 Total phenolic assay

The assay was performed as described by Ivanov et al. [13] with some modifications.

We used 180 μL of freshly prepared Folin-Ciocalteu reagent (5.0 mL Folin – Ciocalteu (Sigma, Germany) and 45.0 mL ddH_2O) and 20 μL sample. After 5 min. of incubation, 100 μL sodium carbonate (7.5% v/w) were added. The light absorbance was measured at 750 nm after 30 min incubation at 21°C in darkness on Multiscan FC microplate reader (ThermoScientific, USA). The results were expressed as μg gallic acid equivalents (GAE) per g dry weight (DW), according to a calibration curve, built in the range of 0.05 - 3 mg/L gallic acid (Sigma, Germany).

2.7 Statistical analysis

Microsoft Excel, SigmaPlot 10.0, MINITAB 14 were used for statistical analyses.

3 Results

3.1 Cultivation of *Gardenia jasminoides* shoot cultures in temporary immersion systems (TIS)

Cultivation of *G. jasminoides* shoot was performed in a 200 ml working volume in TIS at 24°C, in the dark for 30 d. The cultivation regimes included 15 min. immersion every 4, 8 and 12 h representing cultivation regime 1, 2 and 3 respectively. For the experiments 7 g of shoot cultures were used. Figs. 1, 2 and 3 show *G. jasminoides* shoot cultures representing the three different regimes of TIS cultivation.



Fig. 1. Shoot cultures of *Gardenia jasminoides*, grown in Temporary immersion systems (TIS) under cultivation regime 1 (15 min immersion every 4 h)



Fig. 2. Shoot cultures of *Gardenia jasminoides*, grown in Temporary immersion systems (TIS) under cultivation regime 2 (15 min. immersion every 8 h)

The results showed that the highest content of dry biomass was achieved using regime 2 of TIS cultivation with 15 min immersion every 8 h (Fig. 4).



Fig.3. Shoot cultures of *Gardenia jasminoides*, grown in Temporary immersion systems (TIS) under cultivation regime 3 (15 min immersion every 12 h)

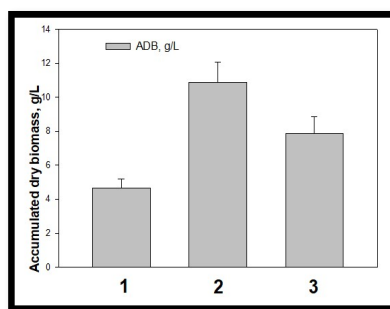


Fig. 4. Accumulated dry biomass (ADB), g/L, from shoot culture *Gardenia jasminoides* grown in temporary immersion systems (TIS):

- 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

The highest GIDW of *G. jasminoides* shoots, cultivated in TIS was recorded for on regime 2 with 15 min. immersion every 8 h (Fig 5).

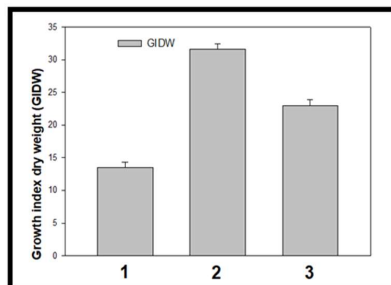


Fig. 5. Growth index dry weight (GIDW), of *Gardenia jasminoides* shoot cultures grown in temporary immersion systems (TIS) under different regimes of cultivation: 1 – Regime 1- 15 min immersion every 4 h; 2 – Regime 2 - 15 min immersion every 8 h; 3 – Regime 3 - 15 min immersion every 12 h

3.2 Phytochemical profile of methanolic extracts from *Gardenia jasminoides* Ellis shoot cultures grown, under different regimes of cultivation

There are no significant differences in the results of methanolic extracts from *G. jasminoides* Ellis shoot cultures grown in TIS under different regimes of cultivation. For example, the content of salicylic acid ($313.62 \pm 38.17 \mu\text{g/g DW}$, $284.49 \pm 117.04 \mu\text{g/g DW}$ and $327.86 \pm 118.12 \mu\text{g/g DW}$ respectively for regimes 15 min. immersion on every 4 h, 15 min immersion on every 8 h, 15 min immersion on every 12 h) in the three regimes are statistically insignificant (Table 1).

The shoot culture produced maximal amounts of rosmarinic acid ($559.19 \pm 19.85 \mu\text{g/g DW}$) and rutin ($891.67 \pm 122.29 \mu\text{g/g DW}$) when cultivated on regime 3 (15 min immersion on every 12 h) (Table 1).

Table 1 HPLC quantitation of polyphenolics in methanolic extract from *Gardenia jasminoides* shoot cultures grown under different regimes of cultivation= Regime 1 (15 min immersion every 4 h); Regime 2 (15 min immersion every 8 h); Regime 3 (15 min immersion every 12 h)

	Regime 1, $\mu\text{g/g}$	Regime 2, $\mu\text{g/g}$	Regime 3, $\mu\text{g/g}$
Chlorogenic acid	$123.83 \pm 18.93^{**}$	158.84 ± 15.34^a	164.82 ± 32.36^a
Caffeic acid	23.13 ± 5.9^a	18.83 ± 6.68^a	26.90 ± 6.80^a
Ferulic acid	103.06 ± 10.97^a	55.56 ± 3.16^a	39.47 ± 4.51^a
Salicylic acid	313.62 ± 38.17^a	284.49 ± 117.04^a	327.86 ± 118.12^a
Rutin	519.65 ± 11.11^b	126.85 ± 0^c	891.67 ± 122.29^a
Hesperidin	126.72 ± 0^b	132.94 ± 6.07^a	130.90 ± 10.55^a
Rosmarinic acid	355.24 ± 38.25^b	558.48 ± 2.69^a	559.19 ± 19.85^a
Quercetin	204.48 ± 16.63^a	306.46 ± 77.25^a	60.10 ± 13.91^a
Kaempferol	22.84 ± 2.28^a	37.47 ± 8.61^a	11.09 ± 2.71^a

*Data presents means \pm SD, n = 3; Means that do not share a letter are significantly different ($p < 0.05$, ANOVA with Tukey pairwise comparisons of means)

3.3 Antioxidant activity analyzes

The results in Table 2 clearly showed that the measured antioxidant activity of the methanolic extracts obtained from shoots grown under different cultivation regimes in TIS were not significant differences. A statistical analyses showed correlation at shoots grown under regime 3 between CUPRAC ($r = 0.816$), FRAP ($r = 0.823$), DPPH ($r = 0.754$), ABTS ($r = 0.743$) and accumulation of total phenolic. Opposite in regime 1 and regime 2 the correlation was lower.

4 Discussion

The use of plant cultures as a matrix for the production of cosmetic product ingredients is intriguing for large part of the scientific community. Finding optimal conditions for growth and production of metabolites is the first step in scaling up the process. For example, the company Mibelle Biochemistry Switzerland recently developed a liposomal preparation based on extract from apple stem cells which has a protective effect over human skin and hair follicles. They also developed products with active ingredients from plant *in vitro* cultures like facial creams, facial serums, eye creams, facial masks, hair oils, make-up products [14].

Table 2 Antioxidant activity and total phenolic content of methanolic extracts from *Gardenia jasminoides* shoot cultures grown under different regimes of cultivation. Regime 1 (15 min immersion every 4 h); Regime 2 (15 min immersion every 8 h); Regime 3 (15 min immersion every 12 h)

	Regime 1	Regime 2	Regime 3
CUPRAC, $\mu\text{M TE/g DW}$	$39.66 \pm 5.8^{**}$	37.60 ± 5.71^a	48.60 ± 9.56^a
FRAP, $\mu\text{M TE/g DW}$	14.10 ± 0.5^a	13.23 ± 1.49^a	14.14 ± 0.56^a
DPPH, $\mu\text{M TE/g DW}$	18.45 ± 1.91^b	21.32 ± 1.14^a	18.32 ± 6.56^b
ABTS, $\mu\text{M TE/g DW}$	10.73 ± 6.26^a	12.47 ± 1.7^a	11.73 ± 3.90^a
Total phenolic assay, $\mu\text{M GAE/g DW}$	2.25 ± 0.19^a	2.28 ± 0.57^a	2.32 ± 0.52^a

*Data presents means \pm SD, n = 3; Means that do not share a letter are significantly different ($p < 0.05$, ANOVA with Tukey pairwise comparisons of means)

The aim of the present study was to optimize the immersion periods for the best growth and accumulation of polyphenols by *Gardenia jasminoides* shoot cultures. Our results clearly showed that when comparing the effect of different regimes of cultivation in TIS, regime 2 (15 min immersion every 8 h) showed the highest results for ADB and GIDW (Fig. 4 and Fig. 5). Similar thinks we found in this paper by Ivanov et al. [15] where different immersion frequency had significant influence on alkaloids biosynthesis by *L. aestivum*. The influence of the immersion periods on biomass production for achieving the optimal conditions for biomass growth and production of secondary metabolites was investigated.

The maximal contents of norgalanthamine and galantamine were produced on regime with 15 min immersion every 8 h [15].

This paper also investigates the accumulation of polyphenols in the biomass of *Gardenia jasminoides* shoot cultures. The immersion periods had significant effect on the production of secondary metabolites. For example, rutin was found with the highest content under regime 3 ($891.67 \pm 122.29 \mu\text{g/g}$) and the lowest under regime 2 ($126.85 \pm 0 \mu\text{g/g}$). The rosmarinic acid had insignificant results for regime 2 ($558.48 \pm 2.69 \mu\text{g/g}$) and regime 3 ($559.19 \pm 19.85 \mu\text{g/g}$), and lowest content for regime 1 ($355.24 \pm 38.25 \mu\text{g/g}$). The results for hesperidin are statistically insignificant between regime 2 ($132.94 \pm 6.07 \mu\text{g/g}$) and regime 3 ($130.90 \pm 10.55 \mu\text{g/g}$).

Hesperidin and rutin are flavonoids that have a strong anti-inflammatory effect [16]. Rutin and rosmarinic acid (RA) are often used for cosmetic products because it has been shown to possess high antibacterial and antioxidant activity [17],[18]. RA also had anti-aging, anti-allergy, anti-inflammatory activities and were used in skin care products [15].

5 Conclusion

In this study was demonstrated that *G. jasminoides* shoot cultures had significant differences in accumulation of biomass, total phenolic content and antioxidant activity when the plant is cultivated under different regimes.

G. jasminoides shoot cultures showed the best accumulation of biomass cultivated under regime with 15 min immersion on every 8 h. Opposite when shoot cultures from *G. jasminoides* are cultivated on regime with 15 min immersion on every 12 h had the best accumulation of phenolic components and antioxidant activity.

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