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# Qualitative and Quantitative Analysis of *Astragalus trojanus* Batch Cultures with the Effect of Potassium Nitrate, Glucose, Light and Temperature

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***Abstract -*** *In this study, the nitrogen and carbon sources, light conditions and higher temperature was applied to batch cultures of Astragalus trojanus Stev. Callus obtained in semi-solid WPM medium supplemented with 1 mg/L 6-benzyladenine and 100 µg/L selenium were transferred to liquid medium with the same composition under dark conditions. In order to investigate the effects of medium composition and culture conditions on biomass, astragaloside IV and cycloastragenol accumulations, 505 mg/L $KNO_3$, 16 g/L glucose, light conditions (1000 lux) and 28°C temperature were implemented to cell cultures. Biomass did not ascend, however, accumulation of astragaloside IV (AST IV) and cycloastragenol (CG) was affected. The highest AST IV (0.3018 µg/mg) and CG (13.160 µg/mg) concentrations were detected in light conditions and $KNO_3$ added medium, respectively. Addition of $KNO_3$ instead of $NH_4NO_3$ increased cycloastragenol accumulation, but astragaloside IV decreased. At 28°C, neither of the components can be detected.*

***Keywords -*** *Astragalus trojanus, astragaloside IV, batch culture, cell suspension,cycloastragenol*

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## I. INTRODUCTION

*Astragalus trojanus* Stev. (Fabaceae) is an endemic medicinal plant and member of the *Astragalus* genus which is represented by 445 species (224 are endemic) in the flora of Turkey [1,2]. Aqueous root extracts of some *Astragalus* species are used in Turkish folkloric medicine as an antiperspirant, diuretic, tonic, wound-healing drug and for the treatment of diabetes mellitus, nephritis, leukemia, and uterine cancer [3].

*Astragalus* genus contains triterpene saponins which are thought to be effective in immune system stimulation, and have anticancer and wound healing effects [4]. Mainly, three major classes of compounds, polysaccharides, saponins and isoflavonoids were isolated from Astragalus species [5]. It has been reported that six new compounds belonging to cycloartane glycosides were isolated from the the roots of Astragalus trojanus [6].

*Astragalus* genera are the richest source of cycloartanes, the unique triterpenoids with a characteristic 9,19-cyclopropane ring, and cycloastragenol is a key intermediate in the biosynthesis of different phytosterols [7]. It has been shown that cycloastragenol (CA), the main aglycon of many cycloartane-type glycosides, extends T cell proliferation by increasing telomarase activity which helps the delay the onset of cellular aging and pharmacological telomerase-based therapy enhances immune function [8]. CA has been introduced to the dietary supplement market as a new antiaging entity. As the content of this compound in Astragalus species is very low, ranging between 0.1% and 0.5%, and transformation of the glycosides into their aglycone (CA) is problematic due to acid labile property of the cyclopropane ring, new methodologies must be established to satisfy the increasing demand for these high value added compounds. It is also reported that AST IV was listed in the 2005 edition of Pharmacopoeia of the People's Republic of China [9,10]**.**

After 1970's, it was realized that plant cell cultures could produce the secondary metabolites at high concentrations [11]. Today, plant cell cultures are one of the promising source for secondary metabolite production at high concentrations and this technique is also used to investigate secondary metabolism pathways. In order to enhance production of secondary metabolites via plant cell culture techniques, many biotechnological strategies such as manipulation of nutrients, modification of liquid media, optimizing the culture environment, biotransformation and immobilization have been used [12,13].

In recent studies biotransformation of CA was achieved by microbial transformation methods [7], but cycloartane-type sapogenols can also be produced by tissue culture methods [14,15].

Despite their importance, there has been only a limited number of reports about plant tissue cultures of *Astragalus* genus and most of them are about shoot and organ regenerations and hairy root cultures [14-17]. In our previous study, we have found 3.5 µg/mg astragaloside IV (AST IV) and 4.8 µg/mg CA in callus tissues regenerated from the stems of *A. trojanus* in dark conditions [10].

The aim of this study is to investigate the effects of potassium nitrate, glucose, light and temperature on AST IV and cycloastragenol accumulations in batch cultures of *A. trojanus*.

## II. MATERIALS AND METHODS

### 2.1. Plant Material and Medium Preparation

Stems of 4-week-old *in vitro* micropropagated plantlets grown in semi-solid WPM [18] medium supplemented with 1 mg/L 6-benzyladenine (BA), 3% sucrose and 0.7% agar were cut into 1 cm segments and placed into semi-solid WPM media supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 100 µg/L selenium, doubled concentration of WPM vitamins, %0.7 agar, %3 sucrose (WD3SV). The pH of the medium was adjusted to 5.8. The media were autoclaved for 15 minutes at 121ºC and a pressure of 1.2 kg/cm$^3$. Stem explants were incubated under dark conditions at 24±1ºC. 2 g of four week old calli obtained from stem explants on WD3SV media were inoculated into 50 mL liquid medium, with the same composition except agar, in 250 mL flasks with three replicates. They were cultivated on an orbital shaker at 100 rpm and 24±1ºC under dark conditions. After four weeks, suspension cultures were diluted to their half concentration with liquid WD3SV medium and subcultured two times with the intervals of 14 days. After the second subculture, batch cultures were established with inoculation of suspension cultures grown in WD3SV medium into the modified media listed in **Table 1.**

### 2.2. Growth Parameters of Suspension Cultures

In the fourth week of culture, living cell and total cell numbers of suspension cultures grown in WD3SV medium were determined using trypan blue. The viability percentage of culture (living cell number per ml/ total cell number per ml x 100) was also determined. At the end of culture period, the biomass was filtered through normal filter paper to identify the total fresh weights of biomass for each flask. After drying, dry weights (g/L) and dry weight percentages (%) (dry weight x 100 /fresh weight) were calculated.

**Table 1: Contents of batch culture media inoculated with suspension cultures grown in WD3SV medium.**

| Medium Code | WD3SV-control | WD3SV - KNO3 | WD3SV - Glucose | WD3SV – Light (1000 lux) | WD3SV - 28C |
|---|---|---|---|---|---|
| Basal Medium | WPM | WPM | WPM | WPM | WPM |
| Nicotinic Acid (mg/L) | 1 | 1 | 1 | 1 | 1 |
| Pyridoxine-HCl (mg/L) | 1 | 1 | 1 | 1 | 1 |
| Tiamin-HCl (mg/L) | 2 | 2 | 2 | 2 | 2 |
| Selenium (µg/L) | 100 | 100 | 100 | 100 | 100 |
| Sucrose (g/L) | 30 | 30 | 0 | 30 | 30 |
| Glucose (g/L) | 0 | 0 | 16 | 0 | 0 |
| $NH_4NO_3$ (mg/L) | 400 | 0 | 400 | 400 | 400 |
| $KNO_3$ (mg/L) | 0 | 505 | 0 | 0 | 0 |
| Culture Condition | dark | dark | dark | light (1000 lux) | dark |
| Temperature (°C) | 24±1 | 24±1 | 24±1 | 24±1 | 28±1 |
| pH | 5.8 | 5.8 | 5.8 | 5.8 | 5.8 |
| 2,4-D (mg/L) | 1 | 1 | 1 | 1 | 1 |

### 2.3. Sample Preparation and HPLC-ELSD Method

All the samples were extracted for 15 minutes with 5 mL HPLC grade methanol for 3 times. After sonication, samples were centrifuged until clear extracts were obtained. All of the clear extracts were combined and evaporated under vacuum. Evaporated samples were lyophilisated to get dry extracts. Dried extracts were dissolved with HPLC grade methanol to obtain concentration of 5 mg/mL. All the sample solutions were passed through 0.45 nylon membrane filters prior to injections.

HPLC-ELSD analyses were performed on a Thermo Surveyor Plus instrument, equipped with quaternary pump, autosampler, column oven, diode array (Thermo Fisher Scientific, MA, USA) and Softa 300S ELSD detector (SofTA Corporation, CO, USA). For all separations a Thermo Hypersil GOLD RP (100x4.6 mm, 5 µm particle size; Thermo Fisher Scientific, MA, USA) HPLC column was used. LC separations were carried out using following solvents: water (A) and acetonitrile (B) and gradient elution was performed as: 0 min 72A/28B, in 5 min to 70A/30B, in 4 min to 38A/62B, in 3 min to 30A/70B hold for 3 min. Additionally, column was washed with 5A/95B for 2.5 min and prior to the next injection the column was equilibrated for 2.5 min with the beginning conditions. Detection was performed with ELSD detector with the settings as: 40ºC at

spray chamber, 70ºC at operating chamber, 105ºC at drift tub and $N_2$ pressure 50 psi. Flow rate was 2 mL/min, column temperature was 30ºC and injection volume was 10 µL.

Two main compounds of *Astragalus* species, astragaloside IV (AST IV) and cycloastragenol (CA), were calibrated for quantitative analysis of samples. Standard stock solutions were prepared with methanol (2000 µg/mL) and additinonal six levels were prepared by dilution of stock solutions (1000 µg/mL, 500 µg/mL, 250 µg/mL, 100 µg/mL, 62.5 µg/mL, 25 µg/mL) with methanol. Retention times for AST IV was 7.22 min and CA was 11.23 min. Regression coefficent for AST IV was 0.9958 and for CA was 0.9973.

### 2.4. Statistical Analyses

Experiments were implemented in a factorial randomized plots design with three replicates. Data were analysed with one way ANOVA test and post hoc LSD tests were performed.

## III. RESULTS

Some *Astragalus* species are found on seleniferous soils and they could accumulate several thousand ppm of selenium [19]. Selenium may cause inhibition of callus regenerations. Accumulator species formed calli at high concentrations of selenate and selenite, however calli regeneration was inhibited in nonaccumulator species [20]. Vitamin requirement in plant cell and tissue cultures shows variations according to the nature of plant and the type of culture [21]. In our study, living ($5.23x10^6$) and total cell numbers/mL ($5.43x10^6$) and cell viability (96.13%) were enhanced with the addition of selenium (100 µg/L) and doubled concentration of WPM vitamins (**Table 2**). These results showed that addition of 100 µg/L selenium and doubled concentration of WPM vitamins had positive effect on cell growth.

**Table 2: Living and total cell numbers/mL and viability of batch cultures.**

| **WD3SV medium** | |
|---|---|
| Living Cell Number/ml | $5.23x10^6$ |
| Total Cell Number/ml | $5.43x10^6$ |
| Viability (%) | 96.13 |

Biomass accumulation decreased when $KNO_3$ added to WD3SV medium instead of $NH_4NO_3$ (**Table 3**). However, 505 mg/L $KNO_3$ (WD3SV – KNO3 medium) stimulated CG accumulation and CG concentration increased from 0.1744 µg/mg to 13.160 µg/mg, which means approximately 75 times higher than control (**Table 4**). It was also the highest concentration of CG amongst the media tested. On the other hand, AST IV can not be detected in the biomass of WD3SV – KNO3 mediumWhen sucrose was replaced with glucose in WD3SV medium, fresh and dry weights and dry weight percentages showed a significant decrease (**Table 3**). Moreover, AST-IV can not be detected and CG concentration decreased from 0.1744 µg/mg to 0.1119 µg/mg (**Table 4**).

Biomass was decreased due to exposure of light and higher temperature. Maximum AST IV content (0.3018 µg/mg), which was two times higher than control, was detected under light conditions, however, CG concentration was decreased from 0.1744 µg/mg to 0.0046 µg/mg. At 28°C, AST IV and CG can not be detectedLight and temperature are effective factors on biomass yield and secondary metabolite production (**Table 3** and **4**).

**Table 3: Fresh (FW) and dry (DW) weights (g), dry weight percentages (DWP) (%) of batch cultures in WD3SV media in the fourth week of culture.**

| **Medium Code** | **FW* (g/L)** | **DW** (g/L)** | **DWP (%)*** ** |
|---|---|---|---|
| WD3SV-control | 4.17±0.09a | 1.68±0.20a | 40.23±4.78a |
| WD3SV-KNO3 | 0.0280±0.0064b | 0.0037±0.0002b | 13.10±3.3607b |
| WD3SV-Glucose | 0.0249±0.009b | 0.0039±0.0016b | 15.80±0.948b |
| WD3SV-Light | 0.0086±0.002b | 0.0014±0.0005b | 15.09±1.79b |
| WD3SV-28C | 0.0085±0.0007b | 0.0012±0.0001b | 13.59±0.55b |

Values within column followed by different small letters are significantly different at the 0.01 level by LSD's test. *p<0.01; F:2262.146; MSE:0.005; LSD:0.175 **p<0.01; F:69.464; MSE:0.024; LSD:0.403 ***p<0.01; F:17.130; MSE:23.053; LSD:14.423

**Table 4: AST IV and CG concentrations of batch cultures.**

| **Medium Code** | **AST IV (µg/mg)** | **CG (µg/mg)** |
|---|---|---|
| WD3SV-control | 0.1558 | 0.1744 |
| WD3SV-KNO3 | 0.0000 | 13.160 |
| WD3SV-Glucose | 0.0000 | 0.1119 |
| WD3SV-Light | 0.3018 | 0.0046 |
| WD3SV-28C | 0.0000 | 0.0000 |

$KNO_3$, glucose, light conditions and higher temperature did not enhanced biomass of suspension cultures, however, it was shown that these factors had effects on secondary metabolism of *Astragalus trojanus* cell cultures. Maximum AST IV (0.3018 μg/mg) and CG (13.160 μg/mg) contents were detected in WD3SV-Light and WD3SV-KNO3 media, respectively. AST IV contents of the native plant were 36.2 μg/mg in roots and 30.9 μg/mg in stems, whereas CA contents were 3 μg/mg in roots and 2.3 μg/mg in stems. **Ionkova et al. (1997)** reported that astragaloside contents (Astragalosides I, II and III) of hairy root cultures of *Astragalus mongholicus* were approximately 2% of dry weight (20 μg/mg) [14]. In *Astragalus membranaceus* hairy root cultures, AST IV amount was found 1.4 mg/g [15]. In *Radix Astragali (A. membranaceus* var. *mongholicus*), the average content of AST-IV was found to be 0.016% (0.16 μg/mg) [22].

## IV. CONCLUSION

Data obtained in this study showed that AST IV and CG contents of *A. trojanus* cell cultures can be manipulated by different medium compositions and culture conditions. Different light intensities, nitrogen and carbon sources and temperature can be implemented to cell cultures of *A. trojanus* in order to enhance biomass and secondary metabolite accumulation.

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