Avrupa Bilim ve Teknoloji Dergisi
Sayı 14, S. 272-279, Aralık 2018

Araştırma Makalesi

www.ejosat.com ISSN:2148-2683

European Journal of Science and Technology
No. 14, pp.272-279, December 2018

Research Article

Optimization of Precursor and Elicitor Utilization in Batch Cultures of *Astragalus trojanus* Stev.

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(First received 21 September 2018 and in final form 30 November 2018)

(DOI: 10.31590/ejosat.461308)

Abstract

Elicitor and precursor applications are commonly used to induce secondary metabolism in plant cell cultures. In this study, methyl jasmonate, jasmonic acid, salicylic acid and pectin were used as elicitors and β-sitosterol was used as a precursor in batch cultures of *Astragalus trojanus* in order to trigger astragaloside IV and cycloastragenol productions. Growth parameters (fresh and dry weights and dry weight percentages) of batch cultures were also evaluated in order to understand the effects of elicitors and precursor on primary metabolism. All elicitors and precursor used in this study triggered metabolite production at different stages of culture period. The highest astragaloside IV accumulation (0.9435 µg/mg) was detected in medium supplemented with 50 µM methyl jasmonate at the 14th day of culture period, whereas the highest cycloastragenol concentration (0.3626 µg/mg) was found in medium supplemented with 50 µM jasmonic acid at the 28th day of culture period. Large scale cultivation was also performed and 0.3759 µg/mg astragaloside IV was detected in medium supplemented with 50 µM methyl jasmonate at the 14th day.

Key words: *Astragalus trojanus*, batch culture, elicitor, large-scale production, precursor, primary and secondary metabolism.

1. Introduction

Astragalus genus contains triterpene saponins which are thought to be effective in human immune system stimulation, and have anticancer and wound healing effects **(Bedir et al. 2000a; Hasançebi et al. 2011; Boroujerdnia et al. 2011)**. Mainly, three major classes of compounds, polysaccharides, saponins and isoflavonoids were isolated from *Astragalus* species **(Nalbantsoy et al. 2011)**. It has been reported that six new compounds belonging to cycloartane glycosides were isolated from the the roots of *Astragalus trojanus* **(Sevimli-Gür et al., 2011)**.

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 **(Nalbantsoy et al. 2012)**. 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 **(Valenzuela et al. 2009)**. 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 astragaloide IV (AST IV) was listed in the 2005 edition of Pharmacopoeia of the People's Republic of China **(Wang et al. 2009; Nartop et al. 2013a).** *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 **(Davis 1970; Bedir et al. 2000b)**. 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 **(Savran et al. 2012; Cai et al. 2004)**.

After 1970's, it was realized that plant cell cultures could produce the secondary metabolites at high concentrations **(Misawa 1994)**. Today, plant cell cultures are one of the promising source of secondary metabolite production at high concentrations and this technique is also used to investigate secondary metabolism pathways and to develop metabolic engineering in plant systems. 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 **(Nartop and Gürel 2013; Nartop et al. 2013b)**. Apart from these strategies, precursors and elicitors are utilized in order to enhance and manipulate secondary metabolism in plant cell cultures.

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Precursors and elicitors play important role in biosynthetic pathways of secondary metabolites and increase yield of target substances (**Patel and Krishnamurthy 2013**). 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 **(Turgut-Kara and Arı 2006; Abd El-Mavla 2010)** and organ regenerations **(Erişen et al. 2010; Erişen et al. 2011)** and hairy root cultures **(Ionkova et al. 1997; Du et al. 2003)**. 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 *Astragalus trojanus* in dark conditions **(Nartop et al. 2013a)**. The biotransformation of CA was also achieved by microbial transformation methods, but cycloartane-type sapogenols can also be produced by tissue culture methods (**Ionkova et al. 1997; Du et al. 2003)**.

The aim of this study is to investigate the effects of elicitors such as methyl jasmonate (MeJA), jasmonic acid (JA), salicylic acid (SA), pectin (P) and also β-sitosterol (β) as precursor on astragalosides (AST IV and CA) and biomass accumulations in batch cultures of *Astragalus trojanus*.

2. Materials and Methods

2.1. Establishment of cell cultures

Stems of 4-week-old *in vitro* micropropagated plantlets grown in semi-solid WPM **(Lloyd and McCown, 1980)** medium supplemented with 1 mg/L 6-benzyladenine (BA), 3% sucrose and 0.7% agar were cut into 1 cm segments. They were placed into semi-solid WPM media supplemented with 1 mg/L 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.**

Table 1: Contents of the media used for elicitor and precursor applications in batch cultures.

	Media Code	2,4-D (mg/L)	Sucrose Content (g/L)	pH	Vitamin Content	Selenium Content (µg/L)	Elicitor and Precursor Content
Control	WD3SV	1	30 g/L	5.8	WPM vitamins (2X)	100	--
Elicitors	WD3SV- 50MeJA	1	30 g/L	5.8	WPM vitamins (2X)	100	50 µM MeJA
	WD3SV- 100MeJA	1	30 g/L	5.8	WPM vitamins (2X)	100	100 µM MeJA
	WD3SV-50JA	1	30 g/L	5.8	WPM vitamins (2X)	100	50 µM JA
	WD3SV-100JA	1	30 g/L	5.8	WPM vitamins (2X)	100	100 µM JA
	WD3SV-1SA	1	30 g/L	5.8	WPM vitamins (2X)	100	1 mM SA
	WD3SV-5SA	1	30 g/L	5.8	WPM vitamins (2X)	100	5 mM SA
	WD3SV-5P	1	30 g/L	5.8	WPM vitamins (2X)	100	%0.05 P
	WD3SV-10P	1	30 g/L	5.8	WPM vitamins (2X)	100	%0.1 P
Precursor	WD3SV-100β	1	30 g/L	5.8	WPM vitamins (2X)	100	100 mg/L β
	WD3SV-200β	1	30 g/L	5.8	WPM vitamins (2X)	100	200 mg/L β

2.2. Scaling Up

Suspension cultures grown in WD3SV medium were diluted to their half concentration with WD3SV liquid medium and MeJA was added at 50 µM concentration. Working volume/flask volume ratios were determined as 200/500 mL/mL and 400/1000 mL/mL. These cultures were cultivated on an orbital shaker at 100 rpm and 24±1ºC under dark conditions for 14 days.

2.3. Growth Parameters of Suspension Cultures

At the end of each culture period, the biomass was filtered through normal filter paper to identify 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.

2.4. 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.

2.5. 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.

3. Results and Discussion

Biomass accumulation is one of the factors that gives data about primary metabolism of plant cell cultures. In our study, fresh and dry weights and dry weight percentages (growth parameters) were observed in order to investigate primary metabolism of *A. trojanus* batch cultures. Addition of exogenous phytohormones such as JA, MeJA and SA, trigger defense responses in plants and lead to production of bioactive secondary metabolites through several mechanisms **(Baenas et al. 2014).** MeJA, JA and SA are generally not utilized to affect biomass in plant cell and tissue cultures. However, in this study, we showed that these phytohormones were effective both on biomass (which refers to primary metabolism) and secondary metabolite production. Therefore, it is important to optimize their concentrations. Moreover, culture period was found effective on biomass and secondary metabolite accumulation. Growth parameters showed that biomass increased during the culture period (**Table 2**). When batch cultures were started (control - WD3SV), their fresh weight was 0.78 g/L. At the 14th day, FW was recorded 2.45 g/L (3.14 times higher). At the 28th day, FW was 5.43 times higher than the first day. AST IV accumulation was also increased during culture period in control medium (**Table 3**). AST IV concentration increased 4.65 times and reached to 0.1558 µg/mg from 0.0335 µg/mg at the end of the culture period. CA concentration was 0.1185 µg/mg at the first day and reached its highest concentration at the 14th day (0.2249 µg/mg), however, it decreased to 0.1744 µg/mg at the 28th day.

Table 2: Fresh (FW) and dry (DW) weights (g), dry weight percentages (DWP) (%) of batch cultures in WD3SV (control) medium during culture period.

	Culture Period (Days)		
Growth Parameters	**0**	**14**	**28**
FW (g/L)	0.78	2.45	4.24
DW (g/L)	0.26	0.88	1.68
DWP (%)	34.53	37.73	40.22

Table 3: AST IV and CA contents (µg/mg) of elicitors and precursor applied media at 1st, 14th and 28th days of culture period.

Medium Code	AST IV (µg/mg)			CA (µg/mg)		
	1. day	**14. day**	**28.day**	**1. day**	**14. day**	**28.day**
WD3SV	0.0335	0.0775	0.1558	0.1185	0.2249	0.1744
50MeJA	0.0000	**0.9435**	0.0000	0.0000	0.1930	0.0000
100MeJA	0.2743	0.3434	0.0000	0.0000	0.0000	0.1429
50JA	0.0000	0.0000	0.0000	0.0171	0.0116	**0.3626**
100JA	0.2032	0.2381	0.0000	0.0000	0.0000	0.0000
1SA	0.0000	0.0258	0.0319	0.0582	0.0563	0.0193
5SA	0.0000	0.1052	0.0000	0.0107	0.0446	0.0114
5P	0.2815	0.0237	0.0716	0.0004	0.0189	0.0115
10P	0.0000	0.0799	0.0000	0.0022	0.0102	0.0000
β100	0.0566	0.4748	0.0585	0.0000	0.0186	0.0189
β200	0.0757	0.0000	0.0362	0.0198	0.0088	0.0345

3.1. Effects of MeJA as elicitor

The jasmonates (MeJA and JA) are signal compounds in elicitation process leading to biosynthesis of secondary metabolites in plant cell cultures. MeJA is believed to be hydrolyzed by endogenous esterases to free jasmonic acid (JA) within the plant tissue (**Baenas et al. 2014**). In 50 µM and 100 µM MeJA applications, primary metabolism showed a decrease compared to control medium (WD3SV), however, biomass increased during culture period both in WD3SV-50MeJA and WD3SV-100MeJA (**Table 4**). In WD3SV-50MeJA medium, FW decreased from 2.45 g/L to 1.48 g/L (40% reduction) at the 14th day of culture period. DW and DWP were showed an increase which indicates higher biomass quality. Similarly, at the 28th day of culture period, FW decreased from 4.24 g/L to 3.57 g/L (16% reduction). DW also showed a 12% decrease, however, DWP was increased from 40.22% to 42.39%. In WD3SV-100MeJA medium, FW and DW showed an increase at 4% and 26%, respectively, however DWP decreased at 7% at the 14th day of culture. Although FW decreased from 4.24 g/L to 3.57 g/L (16% reduction) at the 28th of culture period, DW and DWP increased at 29% and 59%, respectively. Interaction between culture period and MeJA concentrations in DW was found significant at $p<0.05$. 28th day of culture period was in the first group and 14th day was in the second group ($F= 4.460$; $MSE= 0.102$; $LSD= 0.602$). Interaction between culture period and MeJA concentrations in DWP was also found significant at $p<0.05$ ($F= 7.061$; $MSE= 135.820$; $LSD= 21.95$).

In WD3SV-50MeJA medium, AST IV (0.9435 µg/mg) and CA (0.1930 µg/mg) accumulations were only detected at the 14th day of culture. InWD3SV-100MeJA medium, AST IV was detected at first and 14th days of culture (0.2743 µg/mg and 0.3434 µg/mg, respectively), however, CA (0.1429 µg/mg) was only detected at the 28th day. As a consequence (**Table 5**), it was shown that 50 µM MeJA addition into WD3SV medium triggered AST IV accumulation in batch cultures. In this medium, CA accumulation was also found at high concentration. **Jalalpour et al. (2014)** reported that cells that were elicitated with exogenous

MeJA may had increased endogenous jasmonate which improved their defence system. In this experiment, it was proved that defence system of *A. trojanus* was elicitated with MeJA at both concentrations, therefore, AST IV and CA accumulation was increased.

3.2. Effects of JA as elicitor

JA addition to batch cultures decreased biomass accumulation compared to control medium. Both in WD3SV-50JA and WD3SV-100JA media, FW decreased at the 14th day, then showed an increase at the 28th day (**Table 6**). Interactions between culture period and JA concentrations in FW was found significant at $p<0.01$ (F= 43.347; MSE= 0.097; LSD= 0.855). The highest FW (1.33 g/L) and DW (0.33 g/L) were obtained in WD3SV-100JA medium at the 28th day. Interaction between culture period and JA concentration in DW was also found significant at $p<0.01$ (F= 242.414; MSE= 0.003; LSD= 0.156) and the highest DW was also obtained in WD3SV-100JA medium at the 28th day.

AST IV can not be detected in WD3SV-50 JA medium, however CA accumulation was observed with the addition of 50 µM JA. The highest CA concentration (0.3626 µg/mg) was obtained from WD3SV-50 JA medium at the 28th day of culture (**Table 3** and **5**). This concentration was approximately two times more than control's content (0,1744 µg/mg). 100 µM JA enhanced AST IV content at first (0.2032 µg/mg) and 14th (0.2381 µg/mg) days, however, AST IV can not be detected at 28th day. Moreover, CA can not be detected in WD3SV - 100JA medium. JA is known as one of the keys for defense gene expression and induces the production of various proteins that provides resistance to plants (**Angelova et al. 2006**). Similar to MeJA, addition of exogenous JA also elicitated defence system of the plant and caused to accumulate more AST IV and CA.

Table 4: Fresh (FW) and dry (DW) weights (g), dry weight percentages (DWP) (%) of batch cultures in WD3SV-50MeJA and WD3SV-100MeJA media during culture period.

	WD3SV-50MeJA Culture Period (Days)			**WD3SV-100MeJA Culture Period (Days)**		
Growth Parameters	**0**	**14**	**28**	**0**	**14**	**28**
FW (g/L)*	0.78±0.02	1.48±0.12	3.57±0.44	0.78±0.02	2.55±0.52	3.57±0.44
DW (g/L)	0.26±0.03	0.95±0.11	1.48±0.05	0.26±0.03	1.11±0.27	2.17±0.21
DWP (%)	34.53±4.09	63.82±1.97	42.39±4.02	34.53±4.09	35.20±6.63	64.32±13.98

*(*p<0.01; F= 38.198; MSE= 0.444; LSD= 1.446)*

Table 5: AST IV and CA contents of batch cultures (Values within column followed by different small letters are significantly different by LSD's test).

Table 5		
Medium Code	**AST IV (µg/mg)***	**CA (µg/mg)****
WD3SV	0.114 cd	**0.173 a**
WD3SV - 50MeJA	**0.943 a**	**0.193 a**
WD3SV - 100MeJA	0.371 b	0.143 ab
WD3SV - 50JA	0.000 d	**0.196 a**
WD3SV - 100JA	0.331 b	0.000 c
WD3SV - 1SA	0.043 d	0.057 bc
WD3SV - 5SA	0.158 bcd	0.022 c
WD3SV - 5P	0.200 bcd	0.020 c
WD3SV - 10P	0.080 cd	0.007 c
WD3SV - β100	0.295 bc	0.028 c
WD3SV - β200	0.084 cd	0.024 c

*(*p<0.05; F = 12.989, HKO = 0.016, LSD = 0.215)*
*(**p<0.05; F = 2.781, HKO = 0.012, LSD = 0.106)*

Table 6: Fresh (FW) and dry (DW) weights (g), dry weight percentages (DWP) (%) of batch cultures in WD3SV-50JA and WD3SV-100JA media during culture period.

Table 6						
	WD3SV-50JA Culture Period (Days)			WD3SV-100JA Culture Period (Days)		
Growth Parameters	**0**	**14**	**28**	**0**	**14**	**28**
FW (g/L)	0.78±0.02	0.40±0.21	0.82±0.22	0.78±0.02	0.58±0.16	1.33±0.12
DW (g/L)	0.26±0.03	0.13±0.06	0.09±0.01	0.26±0.03	0.12±0.02	0.33±0.06
DWP (%)*	34.53±4.09	45.06±13.21	14.34±5.76	34.53±4.09	27.39±11.70	26.04±7.03

*(*p<0.01; F= 12.365; MSE= 96.392; LSD= 19.020)*

3.3. Effects of SA as elicitor

1 mM and 5 mM SA additions enhanced biomass accumulation compared to control. In WD3SV-1SA medium, FW increased from 0.78 g/L to 7.10 g/L at the end of culture period. DW was also enhanced up to 3.8 times (**Table 7**). In WD3SV-5SA medium, FW increased up to 17 times and reached 13.23 g/L. Interaction between culture period and SA concentrations in FW was found significant at p<0.01 (F=151.929; MSE=0.097; LSD=0.855) and the highest FW (13.23 g/L) was obtained in WD3SV-5SA medium at the 14th day. Interactions between culture period and SA concentrations in DW was also found significant at p<0.01 (F=242.414; MSE=0.003; LSD=0.156). Addition of 1 mM SA did not enhance AST IV accumulation compared to control, however, 0.0258 µg/mg and 0.0319 µg/mg AST IV were detected at 14th and 28th days, respectively. Addition of 5 mM SA increased AST IV accumulation at 14th day from 0.0775 µg/mg to 0.1052 µg/mg (1.36 times higher) (**Table 3**). CA content can not be increased with the addition of SA, however, all samples obtained from WD3SV-1SA and WD3SV-5SA media contained CA. The highest concentration (0.0582 µg/mg) was observed at the first day of culture. **Qian et al. (2006)** reported that a novel salicylate derivative, trifluoroethyl salicylate, induced taxuyunnanine production in *Taxus chinensis* cell cultures, however, the cell growth was inhibited, which was in contrast with our findings about biomass accumulation.

Table 7: Fresh (FW) and dry (DW) weights (g), dry weight percentages (DWP) (%) of batch cultures in WD3SV-1SA and WD3SV-5SA media during culture period.

Table 7						
	WD3SV-1SA Culture Period (Days)			WD3SV-5SA Culture Period (Days)		
Growth Parameters	**0**	**14**	**28**	**0**	**14**	**28**
FW (g/L)	0.78±0.02	6.13±2.20	7.10±1.55	0.78±0.02	13.23±1.24	12.26±0.94
DW (g/L)	0.26±0.03	1.22±0.32	0.98±0.28	0.26±0.03	2.54±0.30	2.01±0.03
DWP (%)*	34.53±4.09	15.24±0.23	16.83±0.33	34.53±4.09	17.68±0.55	15.56±1.35

(p<0.01; F= 12.365; MSE=96.392; LSD= 19.020)

Table 8: Fresh (FW) and dry (DW) weights (g), dry weight percentages (DWP) (%) of batch cultures in WD3SV-5P and WD3SV-10P media during culture period**.**

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	WD3SV-5P Culture Period (Days)			WD3SV-10P Culture Period (Days)		
Growth Parameters	**0**	**14**	**28**	**0**	**14**	**28**
FW (%)*	0.78±0.02	16.16±4.02	26.68±6.76	0.78±0.02	4.91±2.84	1.37±0.79
DW (%)**	0.26±0.03	3.28±0.76	0.98±0.59	0.26±0.03	0.63±0.36	0.40±0.00
DWP (%)***	34.53±4.09	20.27±0.42	3.67±2.33	34.53±4.09	12.83±4.28	29.30±8.47

** p<0.01; F= 11.840; MSE= 49.179; LSD=15.016, ** p<0.01; F= 10.305; MSE= 0.550; LSD=1.499, *** p<0.01; F= 26.877; MSE= 39.191; LSD=13.405*

Table 9: Fresh (FW) and dry (DW) weights (g), dry weight percentages (DWP) (%) of batch cultures in WD3SV-100β and WD3SV-200β media during culture period.

Table 9						
	WD3SV-100β Culture Period (Days)			WD3SV-200β Culture Period (Days)		
Growth Parameters	**0**	**14**	**28**	**0**	**14**	**28**
FW (%)	0.78±0.02	1.15±0.44	0.60±0.10	0.78±0.02	4.55±0.30	3.14±0.32
DW (%)	0.26±0.03	0.19±0.06	0.14±0.05	0.26±0.03	0.71±0.10	0.19±0.02
DWP (%)*	34.53±4.09	18.86±2.78	27.01±12.90	34.53±4.09	15.41±1.22	6.36±1.19

*(*p<0.01; F= 12.365; MSE= 96.392; LSD=19.020)*

Table 10: Fresh (FW) and dry (DW) weights (g), dry weight percentages (DWP) (%), AST IV and CA accumulationsof batch cultures in large-scale production**.**

Working Volume/Flask Volume (mL/mL) Medium Code	FW (g/L)	DW (g/L)	DWP (%)	AST IV (µg/mg)	CA (µg/mg)
200/500 - control	0.11	0.030	27.1	0.0000	0.0131
200/500 - 50MeJA	0.0067	0.0103	7.8	0.3759	0.0000
400/1000 - control	0.59	0.1419	21	0.3503	0.0114
400/1000 - 50MeJA	0.1721	0.0557	4.2	0.0668	0.0048

3.4. Effects of P as elicitor

Pectin (P) is a immunostimulating polysaccharide and it is one of the most commonly used elicitor to induce secondary metabolism. Addition of 0.05% P increased FW accumulation during culture period (**Table 8**). FW increased from 0.78 g/L to 16.16 g/L at the 14th day and raised to 26.68 g/L at the 28th day. DW was also increased from 0.26 g/L to 3.28 g/L at the 14th day, however decreased to 0.98 g/L at the 28th day. 0.1% P addition increased FW and DW at the 14th day, but it decreased at the end of the culture period. AST IV accumulation enhanced in WD3SV-5P medium up to 8.4 times at the first day of culture. Other samples obtained from WD3SV - 5P and WD3SV - 10P media also contained AST IV, however, its concentration can not be ascended compared to control. Moreover, CA content was found lower in these two media compared to control (**Table 3** and **5**). In the root cultures of *Agelica gigas* Nakai, decursinol angelate accumulation was found proportional to the increase of phenylalanine-lyase activity with the effect of polysaccharide elicitors (**Cho et al. 2003**).

3.5. Effects of β as precursor

β-sitosterol (β) is one of the principal plant sterols (**El-Sharabasy 2004**) and was used as a cycloartane-type saponin precursor which has end-product inhibition effect in *A. mongholicus* hairy root cultures **(Ionkova et al. 1997).** β enhanced FW accumulation at the 14th day of culture both in 100 mg/L and 200 mg/L concentrations. The highest FW (4.55 g/L) and DW (0.71 g/L) were obtained in WD3SV-200β. DWP were at their highest level at the beginning of the culture (**Table 9**). Interaction between culture period and SA concentrations in FW (F= 34.347; MSE= 0.097; LSD=0.605) and interaction between culture period and SA concentrations in DW were found significant at p<0.01 (F= 24.307; MSE= 0.028; LSD=0.321). In WD3SV-100β medium, AST IV accumulation was increased 1.66 times at the first day (0.0566 µg/mg) compared to control (0.0335 µg/mg). At the 14th day, its concentration increased 8.4 times and reached 0.4748 µg/mg. However, the concentration decreased to 0.0585 µg/mg at the end of the culture. CA accumulation was detected at the 14th and 28th days of culture in this medium. In WD3SV-200β medium, AST IV content was found lower compared to WD3SV-100β medium. At the first day, AST IV concentration was found 0.0757 µg/mg. At the 14th day, AST IV can not be detected. However, at the 28th day, AST IV detected at 0.0362 µg/mg concentration. CA content was started with 0.0198 µg/mg at the first day, however, it decreased to 0.0088 µg/mg at the 14th day and then increased to 0.0345 at the end of the culture period (**Table 3 and 5**). **Ionkova et al. (1997)** reported that sitosterol stimulated saponin production in *A. mongholicus* hairy root cultures. This was similar to our findings about AST IV production in the first two weeks of culture period (**Table 3**).

3.6. Scale up production in batch culture

According to data obtained from our study, WD3SV-50MeJA medium was found optimum for large scale production (**Table 3, 4 and 5a)**. In this medium, AST IV and CA contents were 0.9435 µg/mg and 0.1930 µg/mg at the 14th day, respectively. Moreover, FW (1.48 g/L), DW (0.95 g/L) and DWP (63.82%) values were reasonable and appropriate for large scale production. Therefore, WD3SV-50MeJA medium were used with two working volume/flask volume (mL/mL) ratios; 200/500 and 400/1000 (**Table 10**). In both experiments, FW, DW and DWP were found lower than control. However, in 200/500-50MeJA medium, AST IV content was found 0.3759 µg/mg, whereas it can not be detected in control. CA can not be detected in this medium. In 400/1000-50MeJA medium, AST IV content (0.3503 µg/mg) was lower than control (0.0668 µg/mg). Similarly, CA content (0.0048 µg/mg) was also found higher in control (0.0114 µg/mg). These concentrations were lower than the findings that were obtained from biomass of 250 ml flasks.

4. Conclusion

In this study, methyl jasmonate, jasmonic acid, salicylic acid, pectin and β-sitosterol were used to enhance two cycloartane-type glycosides, AST IV and CA. They elicitors and precursor increased AST IV and CA productions. It was also shown that culture period (**Table 5**) and scaling up were also important factors in production of cycloartane-type glycosides. The highest AST IV content (0.9435 µg/mg) was obtained in WD3SV-50MeJA medium at the 14th day of culture in 250 mL flask, whereas the highest CA content (0.3626 µg/mg) was detected in WD3SV-50JA medium at the 28th day of culture in 250 mL flask. In this study, AST IV contents of the native plant were found 36.2 µg/mg in roots and 30.9 µg/mg in stems, whereas CA contents were found 3 µg/mg in roots and 2.3 µg/mg in stems. **Ionkova et al. (1997)** reported that astragaloside contents (AST I, II and III) of hairy root cultures of *Astragalus mongholicus* were approximately 2% of dry weight (20 µg/mg). In *Astragalus membranaceus* hairy root cultures, AST IV amount was found 1.4 mg/g **(Du et al. 2003)**. In *Radix Astragali (A. membranaceus* var. *mongholicus*), the average content of AST-IV was found to be 0.016% (0.16 µg/mg) **(Li and Fitzloff 2001)**.

A. trojanus contains valuable medicinal compounds, especially in the roots. In this study, we proved that stem-derived cell cultures have a potential of AST IV and CA production in batch cultures under controlled conditions. With this technique, cycloartane-type saponins can be produced in *in vitro* conditions and there will be no need to use the plant in nature. Therefore, different strategies, such as immobilization and application of other stress factors, should be implemented in order to enhance biomass and secondary metabolite production in further studies.

Acknowledgements

The financial supports of The Scientific and Technical Research Council of Turkey (TUBITAK) (Project No. TOVAG-109O627) and *Ege University Science and Technology Centre* (EBILTEM) (BAP-2012/BİL/017) are gratefully acknowledged.

The authors have declared no conflicts of interest.

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