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Astragaloside IV and Cycloastragenol Production Capacity of *Astragalus trojanus* Calli

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Abstract: *Astragalus* species are medicinal plants which produce valuable secondary metabolites, especially cycloartane-type glycosides. In this study, stem and leaf explants of *Astragalus trojanus* were subjected to different plant growth regulators, environmental conditions and media compositions to identify their callus responses. Stem and leaf explants were cultured in Murashige and Skoog (MS) and woody plant (WPM) media supplemented with different concentrations of kinetin, naphthalene acetic acid, 2,4-dichlorophenoxyacetic acid, thidiazurone and indol acetic acid under two light intensities (1000 and 4000 lux) and also in dark conditions. Both MS and WPM media triggered callus regeneration. Although, callus regeneration was observed on both stem and leaf explants, callus biomass accumulation on stem explants were higher. Addition of 100 µg/L selenium and doubled concentration of WPM vitamins enhanced callus biomass on stem explants under dark conditions. Stem explants also regenerated shoots at high frequencies (up to 93%), especially in kinetin added media. Astragaloside IV and cycloastragenol accumulation efficiencies were determined in calli tissues. The highest astragaloside IV production (3.5 µg/mg) was found in callus tissue regenerated from stem explants in D1 medium, whereas the highest cycloastragenol accumulation (4.8 µg/mg) was detected in callus tissue regenerated from stem explants in N2 medium.

Keywords: *Astragalus trojanus*; callus; shoot regeneration; astragaloside IV; cycloastragenol; plant growth regulators.

1. Introduction

Astragalus is the largest genus in the *Fabaceae* family and represented by 445 species (224 are endemic) in the flora of Turkey and 2500-3000 species worldwide [1-4]. *Astragalus* roots are used in traditional chinese medicine due to their antidiabetic, antioxidant and antineoplastic properties [5-6]. Mainly three major classes of compounds, polysaccharides, saponins and isoflavonoids were isolated from *Astragalus* species [2]. *Astragalus* genera are the richest source of cycloartanes, the unique triterpenoids with a characteristic 9,19-cyclopropane. The cycloartanes of *Astragalus* showed wound healing, immunostimulatory, antineoplastic, hepatoprotective, antiperspirant, diuretic, tonic and antiallergic activities [7-13]. A recent discovery has revealed that cycloastragenol (CA), the main aglycon of many cycloartane-type glycosides only found in *Astragalus* genus, extends T cell proliferation by increasing telomarase activity which helps the delay the onset of cellular aging [14]. Indeed, 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

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transformation of the glycosides into their aglycone (CA) is problematic due to acid liability of the cyclopropane ring, new methodologies must be established to satisfy the increasing demand for these high added-value compounds. It is also reported that astragaloside IV (AST IV) was listed in the 2005 edition of Pharmacopoeia of the People's Republic of China [15].

Plant tissue culture techniques have emerged as an alternative approach for the production of valuable secondary metabolites. Cycloartane-type sapogenols can also be produced by plant tissue culture techniques [16-18].

Astragalus trojanus Stev. is an endemic plant mostly found in eastern and central Anatolia (1300-3500 m), central Aegean region and slopes of Toros mountain (1300-2300 m) in Turkey. It has been found as one of the notable species with high cycloartane-type glycoside content [19-21]. Thus as part of our ongoing studies on the Turkish *Astragalus* species, we decided to make an attempt to develop callus culture procedures for *A. trojanus*.

In this study, stem and leaf explants of *A. trojanus* were subjected to different culture conditions and media compositions for callus induction in order to investigate astragaloside IV and cycloastragenol production capacities. Callus and shoot regenerations and browning percentages of explants were also identified.

2. Materials and Methods

2.1. Plant Material and Media Preparation

Stems and leaves of 4-week-old *in vitro* micropropagated plantlets grown in semi-solid WPM [22] 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 MS [23] and WPM media that were listed in Table 1a and 1b. 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 and leaf explants were incubated under two light intensities (1000 and 4000 lux) of 16 h light / 8 h dark photoperiods and also under dark conditions at 24±1ºC.

Media used for callus induction were classified into three groups (Table 1). In the first group, kinetin, naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) were used at 0.1-2 mg/L concentrations and explants were cultivated under light and dark conditions. According to data, obtained from the first media group, the second and third media groups were composed. In the second group higher concentrations of kinetin, NAA and 2,4-D were used and also thidiazurone and indole acetic acid (IAA) were tested. In the third group, explants were exposed to different media compositions; effects of pH changes, sucrose, glucose, NH_4NO_3, vitamin, selenium were investigated. In the second and the third groups, explants were cultivated only in dark conditions (Table 2 and 3).

At the fourth week of cultures, percentages of callus induction, shoot regeneration and browning were determined. Dry weights of callus tissues were recorded and their secondary metabolite analysis were done by HPLC-ELSD method.

2.2. 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

Table 1. Contents of callus regeneration media (a) first and second media groups, (b) third media group.

Table a

	Medium Code	Basal Medium	Growth Regulator Content	Sugar Content	pH	Medium Content
First Group	KIN1	MS	0.1 mg/L KIN	30 g/L sucrose	5.8	-
	KIN2	MS	0.5 mg/L KIN	30 g/L sucrose	5.8	-
	KIN3	MS	1 mg/L KIN	30 g/L sucrose	5.8	-
	KIN4	MS	2 mg/L KIN	30 g/L sucrose	5.8	-
	WK1	WPM	0.1 mg/L KIN	30 g/L sucrose	5.8	-
	WK2	WPM	0.5mg/L KIN	30 g/L sucrose	5.8	-
	WK3	WPM	1 mg/L KIN	30 g/L sucrose	5.8	-
	WK4	WPM	2 mg/L KIN	30 g/L sucrose	5.8	-
	N1	MS	0.1 mg/L NAA	30 g/L sucrose	5.8	-
	N2	MS	0.5 mg/L NAA	30 g/L sucrose	5.8	-
	N3	MS	1 mg/L NAA	30 g/L sucrose	5.8	-
	N4	MS	2mg/L NAA	30 g/L sucrose	5.8	-
	WN1	WPM	0.1 mg/L NAA	30 g/L sucrose	5.8	-
	WN2	WPM	0.5 mg/L NAA	30 g/L sucrose	5.8	-
	WN3	WPM	1 mg/L NAA	30 g/L sucrose	5.8	-
	WN4	WPM	2 mg/L NAA	30 g/L sucrose	5.8	-
	D1	MS	0.1 mg/L 2,4-D	30 g/L sucrose	5.8	-
	D2	MS	0.5 mg/L 2,4-D	30 g/L sucrose	5.8	-
	D3	MS	1 mg/L 2,4-D	30 g/L sucrose	5.8	-
	D4	MS	2 mg/L 2,4-D	30 g/L sucrose	5.8	-
	WD1	WPM	0.1 mg/L 2.4-D	30 g/L sucrose	5.8	-
	WD2	WPM	0.5 mg/L 2,4-D	30 g/L sucrose	5.8	-
	WD3	WPM	1 mg/L 2,4-D	30 g/L sucrose	5.8	-
	WD4	WPM	2 mg/L 2,4-D	30 g/L sucrose	5.8	-
Second Group	DB1	MS	1 mg/L 2,4-D + 1 mg/L BA	30 g/L sucrose	5.8	-
	DB2	MS	2 mg/L 2,4-D + 1 mg/L BA	30 g/L sucrose	5.8	-
	N5	MS	4 mg/L NAA	30 g/L sucrose	5.8	-
	N6	MS	6 mg/L NAA	30 g/L sucrose	5.8	-
	WN5	WPM	4 mg/L NAA	30 g/L sucrose	5.8	-
	WN6	WPM	6 mg/L NAA	30 g/L sucrose	5.8	-
	WD5	WPM	4 mg/L 2,4-D	30 g/L sucrose	5.8	-
	WD6	WPM	6 mg/L 2,4-D	30 g/L sucrose	5.8	-
	WD7	WPM	8 mg/L 2,4-D	30 g/L sucrose	5.8	-
	WD8	WPM	10 mg/L 2,4-D	30 g/L sucrose	5.8	-
	TDZ1	WPM	1 mg/L Thidiazuron	30 g/L sucrose	5.8	-
	TDZ3	WPM	3 mg/L Thidiazuron	30 g/L sucrose	5.8	-
	TDZ5	WPM	5 mg/L Thidiazuron	30 g/L sucrose	5.8	-
	WI4	WPM	2 mg/L IAA	30 g/L sucrose	5.8	-
	I4	MS	2 mg/L IAA	30 g/L sucrose	5.8	-

	Medium Code	Basal Medium	Growth Regulator Content	Sugar Content	pH	Medium Content
Third Group	GK	MS	0.1 mg/L 2,4-D 0.5 mg/L KIN	30 g/L glucose	5.8	MS salts 1 mg/L Nicotinic acid 1 mg/L Pyridoxine-HCl 10 mg/L Thiamine-HCl 1.6 mg/L $MgCl_2.6H_2O$
	GK1	MS	0.5 mg/L 2,4-D 1 mg/L BA	80 g/L sucrose	6.5	3800 mg/L KNO_3 12.4 mg/L H_3BO_3 340 mg/L KH_2PO_4 0.5 mg/L $Na_2MoO_4.2H_2O$ 2.5 mg/L Nicotinic acid 2.5 mg/L Pyridoxine-HCl 0.5 mg/L Thiamine-HCl 4 mg/L Glycine
	GK2	MS	0.5 mg/L 2,4-D 1 mg/L BA	30 g/L glucose	6.5	3800 mg/L KNO_3 12.4 mg/L H_3BO_3 340 mg/L KH_2PO_4 0.5 mg/L $Na_2MoO_4.2H_2O$ 2.5 mg/L Nicotinic acid 2.5 mg/L Pyridoxine-HCl 0.5 mg/L Thiamine-HCl 4 mg/L Glycine
	1/5 MS	MS	1 mg/L 2,4-D	30 g/L sucrose	5.8	1/5 strenght MS
	1/10 MS	MS	1 mg/L 2,4-D	30 g/L sucrose	5.8	1/10 strenght MS
	3MS	MS	0.5 mg/L NAA 3 mg/L BA	30 g/L sucrose	5.8	-
	4MS	MS	0.5 mg/L NAA 4 mg/L BA	30 g/L sucrose	5.8	-
	$1NH_4NO_3$	WPM	0.5 mg/L 2,4-D 0.5 mg/L BA	30 g/L sucrose	5.8	1650 mg/L NH_4NO_3
	$2NH_4NO_3$	WPM	0.5 mg/L 2,4-D 0.5 mg/L BA	30 g/L sucrose	5.8	3300 mg/L NH_4NO_3
	$4NH_4NO_3$	WPM	0.5 mg/L 2,4-D 0.5 mg/L BA	30 g/L sucrose	5.8	6600 mg/L NH_4NO_3
	$8NH_4NO_3$	WPM	0.5 mg/L 2,4-D 0.5 mg/L BA	30 g/L sucrose	5.8	13200 mg/L NH_4NO_3
	DBB5	WPM	0.5 mg/L 2,4-D 0.5 mg/L BA	30 g/L sucrose	5.8	1650 mg/L NH_4NO_3 1 mg/L Nicotinic acid 1 mg/L Pyridoxine-HCl 10 mg/L Thiamine-HCl 100 µg/L Selenium
	WD3S	WPM	1 mg/L 2,4-D	30 g/L sucrose	5.8	0.5 mg/L Nicotinic acid 0.5 mg/L Pyridoxine-HCl 1 mg/L Thiamine-HCl 100 µg/L Selenium
	WD3SV	WPM	1 mg/L 2,4-D	30 g/L sucrose	5.8	1 mg/L Nicotinic Acid 1 mg/L Pyridoxine-HCl 2 mg/L Thiamine-HCl 100 µg/L Selenium
	WD3SV60	WPM	1 mg/L 2,4-D	60 g/L sucrose	5.8	1 mg/L Nicotinic Acid 1 mg/L Pyridoxine-HCl 2 mg/L Thiamine-HCl 100 µg/L Selenium

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.

2.3. Statistical Analyses

In the first media group, experiments were implemented in a factorial randomized plots design with four factors (culture conditions, basal media, explant types, plant growth regulator concentrations) (Table 2). In the second and third media groups, experiments were set up in a factorial randomized plots design with one factor (media) (Table 3). Data were analysed with one way ANOVA test and post hoc LSD tests were performed.

3. Results and Discussion

Callus induction on both stem and leaf explants in all media tested were observed mostly in the second week of culture. In light conditions, their colours were green, light green and yellow. In dark conditions, calli were mostly light yellow or cream-coloured. Green and cream-coloured calli were in a compact form, but yellow calli were friable.

In the first media group, 0.1 - 2 mg/L kinetin, NAA or 2,4-D were used to induce callus formation in stem and leaf explants. Shoot regenerations were also observed on stem explants and their regeneration percentages were determined as well.

Stem explants cultivated in media supplemented with kinetin, regenerated shoots under light and dark conditions. Our data revealed that there are differences in the effect of the different concentrations of kinetin (Table 2). The highest shoot formation was observed on stem explants on WK3 medium (93.33%) in light condition (4000 lux), whereas the lowest shoot formation was obtained on KIN3 medium in (6.67%) dark conditions. It was also detected that shoot formation was higher in WPM media (43%) supplemented with kinetin compared to MS media (34%). Interaction between culture condition x medium x explant type x kinetin concentrations was found statistically significant in shoot formation ($F=3.593$; LSD = 0.6803; $p<0.05$). Kinetin is one of the most used cytokinins in plant tissue cultures and cytokinins are usually known to make promotion the formation of buds *in vitro* conditions. Similar to our findings, it was showed that kinetin induced shoot regeneration in *Matthiola incana* [24]. Very little callus tissues were also observed on both stem and leaf explants, but callus turned into brown at higher concentrations of kinetin, especially on leaf explants. The highest browning percentages were observed on leaf explants which were cultivated in medium supplemented with kinetin. Browning is often associated with failure of explants survival and inhibits growth. Phenolics mostly cause oxidative browning of explants and act as inhibitory agents [25]. It is showed that kinetin caused more damage on leaf explants compared to stem explants.

Table 2. Percentages of browning, shoot regeneration and callus regeneration of callus cultures from leaf and stem explants under light and dark conditions in first media group (%)

Light / Dark Condition	Explant	Kinetin*			NAA**			2.4-D***			
		Medium Code	Percentage of Browning (%)	Percentage of Shoot Regeneration (%)	Medium Code	Percentage of Browning (%)	Percentage of Shoot Regeneration (%)	Percentage of Callus Regeneration (%)	Medium Code	Percentage of Browning (%)	Percentage of Shoot Regeneration (%)	Percentage of Callus Regeneration (%)
Light (4000 lux)	Leaf	KIN1	90.48±2.75	0.00	N1	66.67±0.58	0.00	0.00 H	D1	20.67±0.33	0.00	0.33±0.33 WX
		KIN2	96.83±1.59	0.00	N2	65.08±1.45	0.00	0.00 H	D2	21.00±0.00	0.00	1.33±0.88 TUVW
		KIN3	96.83±1.59	0.00	N3	60.32±1.77	0.00	0.00 H	D3	21.00±0.00	0.00	5.00±1.53 NOPQ
		KIN4	82.54±9.67	0.00	N4	50.79±2.61	0.00	0.00 H	D4	21.00±0.00	0.00	2.33±0.88 RST
		WK1	87.30±4.20	0.00	WN1	65.08±1.20	0.00	0.00 H	WD1	15.00±0.58	0.00	0.33±0.33 WX
		WK2	95.24±2.75	0.00	WN2	52.38±2.89	0.00	0.00 H	WD2	14.00±2.08	0.00	7.00±2.08 LMN
		WK3	92.06±1.59	0.00	WN3	46.03±4.85	0.00	0.00 H	WD3	10.00±0.58	0.00	10.67±0.88 GHIJ
		WK4	77.78±4.20	0.00	WN4	63.49±1.20	0.00	0.00 H	WD4	4.00±0.58	0.00	15.00±0.00 BCDE
	Stem	KIN1	26.67±6.67	60.00±11.56 BC	N1	84.44±0.88	17.78±1.33 FG	0.00 H	D1	15.00±0.00	2.67±1.20 CD	1.67±0.88 STU
		KIN2	46.67±13.35	53.33±6.67 C	N2	86.66±0.58	6.67±0.00H	2.22±0.33G	D2	15.00±0.00	3.00±1.00 BC	6.33±0.67 HIJKL
		KIN3	57.78±2.22	40.00±11.56 EF	N3	55.56±1.20	22.22±0.33BCD	2.22±0.33G	D3	15.00±0.00	0.33±0.33 JK	3.67±0.88 NOP
		KIN4	77.78±9.70	26.67±6.67 HI	N4	53.33±0.58	26.67±1.16BC	15.56±1.86E	D4	15.00±0.00	0.33±0.34 JK	2.67±0.67 PQR
		WK1	57.78±11.77	37.78±11.77 EFG	WN1	42.22±2.03	6.67±1.00I	0.00 H	WD1	2.00±1.00	5.00±1.00 A	2.00±1.16 QRS
		WK2	53.33±6.67	53.33±13.35 CD	WN2	73.33±0.58	2.22±0.33IJ	0.00 H	WD2	10.00±1.53	1.67±0.33 DEFG	3.67±2.34 TUVW
		WK3	26.67±13.35	93.33±6.67 A	WN3	62.22±1.20	11.11±0.33G	0.00 H	WD3	11.33±1.20	2.00±2.00 GH	2.00±2.00 QRS
		WK4	46.67±17.66	53.33±13.35 CD	WN4	53.33±1.00	46.67±1.00A	0.00 H	WD4	8.00±0.58	0.33±0.33 JK	3.33±1.20 OPQ
Light (1000 lux)	Leaf	KIN1	79.37±6.93	0.00	N1	65.08±1.20	0.00	0.00 H	D1	21.00±0.00	0.00	0.00
		KIN2	95.24±2.75	0.00	N2	52.38±2.89	0.00	0.00 H	D2	17.33±1.77	0.00	3.33±1.45 QRS
		KIN3	92.06±1.59	0.00	N3	46.03±4.85	0.00	0.00 H	D3	18.33±0.88	0.00	1.33±0.67 TUV
		KIN4	80.95±7.28	0.00	N4	63.49±1.20	0.00	0.00 H	D4	19.33±0.67	0.00	1.00±0.00 TUV
		WK1	82.54±4.20	0.00	WN1	85.71±1.16	0.00	0.00 H	WD1	20.00±0.58	0.00	0.67±0.67 VWX

	WK2	90.48±7.28	0.00	WN2	76.19±0.58	0.00	0.00 H	WD2	8.33±0.88	0.00	9.00±1.00 HIJKL
	WK3	63.49±11.12	0.00	WN3	76.19±1.53	0.00	0.00 H	WD3	9.00±2.52	0.00	11.00±2.08 FGHIJ
	WK4	82.54±4.20	0.00	WN4	60.32±1.45	0.00	0.00 H	WD4	2.67±1.33	0.00	16.33±1.33 MNO
	KIN1	33.33±0.00	26.67±0.00 HI	N1	66.67±2.08	11.11±0.33 G	2.22±0.33G	D1	8.33±2.41	3.67±1.20 B	3.00±1.53 PQRS
	KIN2	26.67±0.00	35.56±2.22 EFG	N2	64.44±2.61	4.44±0.67 I	20.00±1.53D	D2	8.33±1.86	2.33±0.67 CDE	4.33±1.20 MNO
	KIN3	13.33±3.85	64.44±2.22 B	N3	40.00±1.00	13.33±0.58 FG	28.89±0.67 C	D3	5.67±0.67	1.33±0.67 FGH	8.00±1.16 FGHI
Stem	KIN4	20.00±3.85	13.33±0.00 J	N4	46.67±1.53	13.33±0.58 FG	15.56±1.45 E	D4	10.00±1.16	1.33±0.88 GH	3.67±0.88 NOP
	WK1	53.33±3.85	22.22±5.89 I	WN1	53.33±3.00	11.11±0.33 G	0.00 H	WD1	8.00±1.53	2.00±1.16 EFG	5.00±0.58 KLMN
	WK2	53.33±6.67	33.33±13.35 EFG	WN2	55.56±0.88	20.00±1.00 DE	0.00 H	WD2	5.00±1.53	2.33±1.20 DEF	7.67±0.33 GHIJ
	WK3	40±11.56	46.67±17.66 DE	WN3	66.67±0.58	17.78±0.33 DEF	0.00 H	WD3	5.33±0.88	0.67±0.67 IJ	9.00±1.00 DEFG
	WK4	48.89±11.12	53.33±13.35 CD	WN4	77.78±0.33	22.22±1.86 CDE	0.00 H	WD4	9.67±2.61	0.67±0.67 IJ	4.67±2.03 MNO
	KIN1	100.00±0.00	0.00	N1	76.19±1.53	0.00	0.00 H	D1	18.67±1.20	0.00	2.33±1.20 STU
	KIN2	100.00±0.00	0.00	N2	60.31±1.45	0.00	0.00 H	D2	9.33±2.41	0.00	11.67±2.41 FGHI
	KIN3	100.00±0.00	0.00	N3	50.79±0.67	0.00	34.92±0.33 B	D3	6.67±2.19	0.00	14.33±2.19 CDEF
Leaf	KIN4	100.00±0.00	0.00	N4	66.67±1.00	0.00	4.76±0.58 F	D4	13.00±0.58	0.00	8.00±0.58 JKLM
	WK1	100.00±0.00	0.00	WN1	84.13±1.86	0.00	0.00 H	WD1	20.00±0.58	0.00	1.00±0.58 UVW
	WK2	100.00±0.00	0.00	WN2	66.67±1.16	0.00	0.00 H	WD2	9.00±2.65	0.00	12.00±2.65 EFGH
	WK3	100.00±0.00	0.00	WN3	92.06±0.88	0.00	0.00 H	WD3	2.00±0.58	0.00	19.00±0.58 AB
Dark	WK4	95.24±2.75	0.00	WN4	79.36±0.88	0.00	0.00 H	WD4	5.33±3.18	0.00	15.67±3.18 ABCD
	KIN1	80.00±0.00	20.00±0.00 I	N1	55.56±0.33	26.67±0.58 B	0.00 H	D1	5.00±1.16	3.00±0.00 BC	7.00±1.16 GHIJK
	KIN2	73.33±6.67	26.67±6.67 HI	N2	57.78±0.88	28.89±0.67 B	4.44±0.33F	D2	0.67±0.67	4.00±1.16 AB	13.00±2.00 ABC
	KIN3	93.33±6.67	6.67±6.67 K	N3	22.22±0.88	26.67±0.58 B	51.11±2.19 A	D3	2.67±0.33	0.00	12.33±0.33 ABC
Stem	KIN4	60.00±0.00	40.00±0.00 E	N4	31.11±0.88	28.89±1.77 B	28.89±0.33 BC	D4	6.67±0.67	0.00	8.33±0.67 EFGH
	WK1	68.89±9.70	31.11±9.70 GH	WN1	75.56±1.67	24.44±1.67 BCD	0.00 H	WD1	5.33±2.61	3.00±1.00 BC	2.67±1.77 QRS
	WK2	73.33±7.71	26.67±7.71 HI	WN2	62.22±0.67	37.78±0.67 A	0.00 H	WD2	6.67±2.61	1.00±0.58 HI	7.33±2.41 GHIJK

WK3	66.67±13.35	33.33±13.35 FGH	WN3	95.56±0.67	4.44±0.67 I	0.00 H	WD3	1.00±0.58	0.333±0.33 JK	**13.67±0.67 A**
WK4	60.00±11.56	40.00±11.56 EF	WN4	84.44±0.67	15.56±0.67 EFG	0.00 H	WD4	7.33±0.33	1.67±0.33 DEFG	6.00±0.00 WX

* $p<0.05$; $F = 3.593$; LSD = 0.6803 (Condition*Medium*Explant*Kin.conc.)

** $p<0.05$; Fcallus = 1.433; Fstem = 1.814; LSDcallus = 0.5610; LSDstem = 0.6761 (Explant*Medium*NAA conc.)/ (Condition*Explant*NAA conc.)

*** $p<0.05$; Fcallus = 1.55; Fstem = 1.443; LSDcallus = 1.056; LSDstem = 0.7793 (Explant*Medium*2,4-D conc.)/ (Explant*2,4-D conc.)

Values within column followed by different capital letters are significantly different at the 0.05 level by LSD's test.

Table 3. Percentages of callus and shoot regenerations on stem explants and percentage of callus regeneration on leaf explants (%)

Medium	Percentage of Callus Regeneration on Stem Explants (%)*	Percentage of Shoot Regeneration on Stem Explants (%)**	Percentage of Callus Regeneration on Leaf Explants (%)***
DB1	-	-	81.25 abc
DB2	-	-	54.17 cde
N5	28.57 fghi	0.00 d	-
N6	76.19 abcde	0.00 d	-
WN5	50.79 bcdefg	0.00 d	-
WN6	41.27 defghi	0.00 d	-
WD5	100.00 a	0.00 d	-
WD6	100.00 a	0.00 d	-
WD7	90.48 ab	0.00 d	-
WD8	92.06 ab	0.00 d	-
TDZ1	22.22 efghi	42.22 a	12.70 h
TDZ3	28.89 fghi	37.77 b	41.27 defgh
TDZ5	0.00 i	37.78 b	30.16 efgh
WI4	0.00 i	22.22 bcd	35.55 defgh
I4	2.22 hi	20.00 bcd	42.22 defgh
GK	66.67 abcdef	8.89 bc	100.00 a
GK1	44.44 abcdef	13.34 bcd	44.45 defg
GK2	62.22 abcdef	6.67 cd	44.44 defg
1/5 MS	35.55 efghi	4.45 cd	20.64 fgh
1/10 MS	37.77 efghi	4.45 cd	15.87 gh
3MS	55.56 bcdef	6.67 cd	36.51 defgh
4MS	38.88 efghi	17.78 bcd	63.49 bcd
$1NH_4NO_3$	28.88 fghi	2.22 bcd	36.51 defgh
$2NH_4NO_3$	66.67 abcdef	22.22 bcd	49.21 def
$4NH_4NO_3$	55.56 bcdef	13.33 bcd	47.62 def
$8NH_4NO_3$	11.11 ghi	4.45 cd	41.27 defgh
WD3S	86.67 abc	0.00 d	52.38 cde
WD3SV	88.89 ab	0.00 d	88.89 ab
WD3SV60	84.44 abcd	6.67 cd	65.08 bcd

* F = 4.808; LSD= 43.786; $p<0.01$
**F= 6.014; LSD= 24.807; $p<0.01$
***F= 7.388; LSD=31.311; $p<0.01$

NAA induced shoot regeneration and callus formation on stem explants. Browning was also observed at high percentages (approximately 65%). It mostly affected leaf explants cultivated in NAA supplemented media. Leaf explants formed callus only in N3 and N4 media. No callus induction was seen on stem explants cultivated in WPM media. However, in MS media, callus formations were observed, except N1 medium. The highest callus induction percentage (51.11%) was found on N3 medium (MS + 1 mg/L NAA) in dark condition. Shoot regeneration was also very high (61.10%) in light and dark conditions despite high browning. Interaction between explant type*medium*NAA

concentration was found statistically significant in callus formation (F=1.433; LSD=0.5610; $p<0.05$). Additionally, interaction between culture condition*explant type*NAA concentration was statistically significant in shoot formation (F=1.814; LSD=0.6761; $p<0.05$). The highest shoot regeneration (46.67%) on stem explants was observed in WN4 medium (WPM + 2 mg/L NAA) in light conditions (4000 lux). NAA is an auxin which is mostly used to regenerate callus *in vitro* conditions. Similar to our findings, it is showed that different NAA concentrations affect callus formation ratios [26-27].

2,4-dichlorophenoxyacetic acid induced callus formation on leaf explants, especially in WPM medium, despite browning. It also induced callus and shoot formation on stem explants. Statistically, interaction between explant type*medium*2,4-D concentration was found significant (F=1.55; LSD = 1.056; $p<0.05$). The highest callus formation ratio (13.67%) was observed in WD3 medium (WPM + 1 mg/L 2,4-D) in dark conditions on stem explants. Interaction between explant type*2,4-D concentration was also significant (F=1.443; LSD=0.7793; $p<0.05$). The highest shoot regeneration (5%) was detected in WD1 medium (WPM + 0.1 mg/L 2,4-D) in light conditions. Different concentrations of 2,4-D affect callus formation ratios. Similar to our findings, 2,4-D was found better for callus induction and biomass accumulation, when compared to NAA [28-29].

In the second media group, callus regeneration percentages were higher (up to 100%) than the first group, especially in WD5 and WD6, which were supplemented with higher concentrations of 2,4-D; however, the increase in biomass was insignificant for both media. When the 2,4-D concentration was increased to 8-10 mg/L, callus regeneration percentages degreased to 90-92%. As mentioned above, media supplemented with 2,4-D caused higher callus regeneartion percentages compared to NAA. In WN5 and WN6 media, callus regeneration percentages on stem explants were 50,79% and 41,27%, respectively. High concentrations of NAA induce root formation [30]. In our study, in WN5 and WN6 media, roots were regenerated from callus tissue in the second week of culture period.

On leaf explants, thidiazurone inducted callus formation at high percentages (41.27%). It also inducted stem explants to regenerate callus tissues as well as shoots. But callus tissue did not grow well and the biomass was lower than expected. IAA stimulated callus induction on stem and leaf explants. Shoot regeneration was also observed on stem explants.

In the third media group, callus inductions were observed on both stem and leaf explants in all media tested. Shoot regenerations were also seen on stem explants. Modifications of WD3 medium (WD3S, WD3SV and WD3SV60) showed high callus induction percentages on stem explants with superior biomass formation. Best biomass growth (0.078 mg) was seen in WD3SV medium (Table 4a). Addition of selenium and doubled concentration of vitamins in WPM medium showed the best results in all media tested in respect to biomass.

Doubled (3300 mg/L) and quadruplicated (6600 mg/L) concentrations of NH_4NO_3 ($2NH_4NO_3$ and $4NH_4NO_3$ medium) also enhanced callus formation. Callus induction percentages on stem explants were increased from 28% ($1NH_4NO_3$ medium) to 67% and 56% ($4NH_4NO_3$ medium) with the effect of higher NH_4NO_3 concentrations, but the highest NH_4NO_3 concentration (13200 mg/L, 8 NH_4NO_3 medium) resulted in decrease in callus formation (11%). Similar effects were also seen on leaf explants' callus induction percentages in these media.

Ghaemi et al. (2011) [31] reported that $MgCl_2$ enhanced callus induction cotton species. In our study, $MgCl_2$ showed similar effect on callus regeneration on stem and leaf explants. 1.6 mg/L $MgCl.6H_2O$ was added to GK medium (66.67%-100%) and callus regeneration percentages were enhanced both on stem and leaf explants compared to GK1 (44.44%-44.45%) and GK2 (62.22%-44.44%) media.

Higher pH value (6.5) did not block callus induction on both stem and leaf explants in GK1 and GK2 media. In GK2 medium, glucose enhanced callus initiation percentages rather than sucrose.

AST IV and CA were detected in callus tissues regenerated from both stem and leaf explants. AST IV and CA contents and dry weights of callus cultures were given in Table 4a and 4b. The highest amount of AST IV was 3.5027 µg/mg in callus tissue regenerated from stem explants on D1 medium. In callus tissue regenerated from leaf explants, the highest AST IV was 1.4483 µg/mg. CA was at highest rate (4.8180 µg/mg) in callus tissue regenerated from stem explants in N2 medium. In WDSV3 medium, AST IV can not be detected, while CA content was 0.0106 µg/mg.

In order to obtain callus from explants of *Astragalus* species, different concentrations of 2,4-D, BA, NAA, TDZ and their binary combinations such as NAA x BA [32], TDZ x NAA [33], 2,4-D x BA [34] were used and very high callus formation ratios were obtained.

Although, callus initiations were observed on both stem and leaf explants incised from *A. trojanus* plantlets in all media tested, biomass accumulation was found very low in our study. To solve this problem, callus tissues were subcultured in the same medium, but they still showed recalcitrancy. Different media compositions (54 media) and different light intensities were tested. In dark conditions, it was observed that callus initiation and biomass accumulation were higher comparing to light conditions, especially on cut edges of stems, which could be attributed to triggered stem cells placed around vascular bundles and absence of negative effect of light on callus initiation and growth [35-36]. As a result of this, callus cultures established in media that belong to second and third groups were cultivated in dark conditions. Callus initiation frequency at 100% was obtained in three media (GK, WD5 and WD6), but the highest biomass accumulation was seen in dark conditions on WD3SV medium (third media group), consisting of 100 µg/L selenium and doubled concentration of WPM vitamins. Some *Astragalus* species grow on selenium rich soils, and they accumulate selenium in high quantities [37]. As a result of vitamins' extremely important role in callus growth [38], doubled concentration of WPM vitamins, in addition to supplemented selenium in medium composition, enhanced callus induction percentages and biomass accumulation.

Table 4. Dry weights (mg), astragaloside IV (AST IV) and cycloastragenol (CA) contents (µg/mg) of callus tissues regenerated from stem (**a**) and leaf (**b**) explants

Table a – Stem Explants

Sample Code	Callus Dry Weights (mg)	AST IV (µg/mg)	CA (µg/mg)
D1	12	35.027	0.2840
D2	15	20.912	0.0000
D3	24	0.0000	0.1450
D4	14	18.696	0.0000
WD1	6	20.891	0.1378
WD2	5	0.0000	0.0000
WD3	14	0.0000	0.0107
WD4	8	0.0000	0.0000
WD3SV	78	0.0000	0.0106
N2	1	0.0000	48.180
N3	5	0.0000	15.051
N4	8	0.0000	0.9006

Table b – Leaf Explants

Sample Code	Callus Dry Weights (mg)	AST IV (µg/mg)	CA (µg/mg)
D1	11	0.0000	0.1537
D2	30	0.0000	0.1099
D3	20	14.483	0.0000
WD1	15	0.8765	0.0000
WD2	15	0.0000	0.1184
WD3	21	0.0000	0.0068
WD4	7	0.4766	0.0000

In our study, the highest amount of AST IV and CA were found to be 3.5027 µg/mg and 4.8180 µg/mg, 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) [18] 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). In *Astragalus membranaceus* hairy root cultures, AST IV amount was found 1.4 mg/g [19]. In *Radix Astragali (A. membranaceus* var. *mongholicus*), the average content of AST-IV was found to be 0.016% (0.16 µg/mg) [39].

Although GK, WD5 and WD6 media had the highest callus initiation percentages (100%), WD3SV medium provided higher biomass accumulation. It was shown that callus tissue of *Astragalus trojanus* has a potential for AST IV and CA production. Although these high value compounds were found at µg/mg level, the yields were comparable with the native plant roots and stems warranting

further studies. Thus our forthcoming studies will be focusing on increment of AST IV and CA production capacity of *A. trojanus* via precursor and elicitor utilizations.

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