African J. Biol. Sci., 18 (2): 1-17 (2022)
ISSN 1687-4870 **e- ISSN 2314-5501 (online)**
www.ajbs.journals.ekb.eg **E.mail: aasdjournal@yahoo.com**

Molecular genetic studies on *Atropa belladonna* L. under the effect of laser radiation and salicylic acid on Atropine, Scopolamine and Hyoscyamine by tissue culture techniques

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Received: March 17, 2022; Accepted: April 1, 2022; Available online: May 12, 2022

ABSTRACT

Atropa belladonna is the most important commercial source of pharmaceutical tropane alkaloids such as Atropine, Scopolamine, and Hyoscyamine. The current study aims to investigate the effects of 6 different helium-neon laser doses (0.0, 10, 15, 20, 25 and 30 J cm^{-2}) and exposure conditions on callus fresh weight and apply biotechnological tools as alternative approach for increasing the accumulation of active agents (atropine) of *A. belladonna* plant using abiotic elicitors salicylic acid precursor feeding. After treatment they were analyzed by a photograph illustrating the banding profile produced by the twelve ISSR primers DNA under the six investigated laser radiation doses and HPLC technique was applied to determine Atropine, Scopolamine and Hyoscyamine contents. The results showed that the highest callus fresh weight, callus dry weight was achieved by callus derived from (leaves and stem) explants, which was cultured on (MS) medium supplemented with different combinations among different concentrations of growth regulators as NAA, 2,4-D, Kin and BAP (at 0.5 and 1.0 mg/l). On the other hand all treatments of callus tissue had affected the content of medically active constituents of *Atropa belladonna*. This study suggests a more efficient method for commercially large-scale production of Atropine, Scopolamine, and Hyoscyamine by *A. belladonna* plants.

Keywords: *Atropa belladonna* L., salicylic acid, callus, laser radiation, ISSR.

INTRODUCTION

Belladonna is a perennial herbaceous plant in the Solanaceae family that is a commercial source of pharmaceutical tropane alkaloids. Scopolamine and Hyoscyamine are produced by the plant and are used as acetylcholine antagonists in both the autonomic and central nervous systems (Guggisberg and Hesse, 1983). Scopolamine is far more useful and valuable for medicinal purposes due to its higher physiological activity and fewer side effects. Low-intensity laser radiation stimulates morphogenetic processes in wild grass tissue cultures, such as rhizogenesis, the formation of morphogenic calli, and the regeneration of plants (Salyaev *et al*., 2001). Because of the physical and biological aspects of laser radiation, its interaction with biological environments has distinct characteristics (Anghel, *et al*. 1999). He–Ne laser preillumination improved salt tolerance in tall fescue seedlings by increasing the expression of antioxidant enzyme genes and the phytochrome B gene (Gao *et al*., 2016). Previous research found that He–Ne lasers had an effect on plant growth and metabolism (Cai *et al*., 2000)

Molecular studies are extremely useful in taxonomic and genetic research (Soller and Beckmann, 1983; Kim *et al*,. 2002; Jacobson and Hedren, 2007; Zietkiewicz *et al*., 1994). All markers have advantages and disadvantages, but in genetically neutral regions, they produce reliable information on genetic diversity (Karp 2002). Electrophoretic techniques have been used to identify and characterize different crop cultivars and to assess the uniformity, purity of agronomic traits of cultivars (Abdel-Tawab *et a l.* 1999a).

The main objective of this study is to investigate the effects of different doses of helium-neon irradiation with time on growth characters of *Atropa belladonna* plant and applying abiotic elicitors salicylic acid, as a biotechnological tool and alternative approach for increasing the accumulation of its active agents of atropine, scopolamine and hyoscyamine. ISSR and HPLC techniques were applied to determine the contents of these agents.

Materials and Methods

Plant materials

Atropa belladonna L. seeds were provided by the Experimental Station of Medicinal Plants, Faculty of Pharmacy, Cairo University, Egypt.

Irradiation

Helium-neon irradiation doses (0.0, 10, 15, 20, 25 and 30 J cm^{-2}) were applied on callus of *Atropa belladonna* plant with a power density of 4.02 mW cm^{-2} at National Institute of Laser Enhanced Sciences (NILES), Cairo University.

Tissue culture experiment:

Initiation of callus from leaves and stem.

Medium supplemented (MS) with different combinations among various concentrations of NAA, 2, 4-D, Kin and BAP (at 0.5 and 1.0 mg/l) (Abd el maksood *et al*. 2017).

Callus production:

Callus induction from shoot tip, cotyledon and hypocotyls segments of *Atropa belladonna* L. cultured on MS with various concentrations of 2,4D or NAA in combinations with BA (Hamad and Jassem, 2011).

Effect of elicitor (salicylic acid) on callus production and active ingredient content:

Is studied according to methods describe by Naik and Al-Khayri (2016).

HPLC Analysis:

The chemical composition of callus of *Atropa belladonna* was studied by means of HPLC analysis under the effect of laser radiation. high-performance liquid chromatography (Gfeller *et al*.,1979) gas liquid chromatography (British pharmacopia, 1993b) and potentiometric titration (British pharmacopeia 1993a).

Chemicals:

Panreac (Spain) supplied chloroform and methanol, Sigma (USA) supplied hyoscyamine and scopolamine as standards, Fluka (Switzerland) supplied ammonium solution 25%, Merck (Germany) supplied sulfuric acid 85-88 %, anhydrous sodium sulphate 99 % and potassium dihydrogenortropospheric acid 99 % and Caledon (Canada) supplied acetonitrile of HPLC grade

Instrumentation:

The extractions were carried out in a power sonic 405 ultrasonic chamber (Hwashin Technologies, Korea). To adjust pH at various stages, a pH-meter model CG-840 (Schott Gerate Gmbh, Germany) was used. HPLC analyses were performed on a C18Lichrospher 100 column (5 m, 250 x 4.6 mm) outfitted with a K-1001 pump, a K-2800 UV-PDA detector, and a 20 l injection loop from Knauer (Germany). The analytical

column was connected to a 10 mm C8 pre-column.

Alkaloid extraction and chromatographic conditions: Materials plants were powdered and then sonicated for 10 min with10 ml of chloroform-methanol-ammonium hydroxide (25%) (15:15:1) per 100 mg of sample.

Calibration curve preparation:

Calibration curves were created by plotting peak areas deferent atropine and scopolamine concentrations, and regression equations were calculated.

Validation of methods:

The method was validated in terms of sensitivity, linearity, precision, and recovery using the International Committee of Harmonization (ICH) guidelines (1996).

HPLC Analysis Procedure:

Tropane alkaloids Hyoscyamine and Scopolamine in the irradiation root lines, non-irradiation root lines and wild type plant roots were extracted and analyzed by HPLC: According methods describe by (Zhang *et al., 2*004).

Molecular genetic studies by ISSR experiments: Isolation of DNA:

The extraction of DNA was performed using DNeasy Mini Kit (QIAGEN) according to (Williams *et al*., 1990).

PCR reactions:

The DNA amplifications were performed in an automated thermal cycle (model Techno 512) programmed for one cycle at 94º C for 4 min followed by 45 cycles of 1 min at 94º C, 1 min at 50º C, and 2 min at 72º C, the reaction was finally stored at 4º C for 10 min. using twelve ISSR primers (ISSR-1, ISSR-2,ISSR-3, ISSR-4, ISSR-6, ISSR-7, ISSR-8, ISSR-9, ISSR-10, ISSR-11, ISSR -12 and ISSR-13) (Joshi *et al*., 2000).

Analysis of data:

The pair wise deference matrix and the phylogram tree among the cultivars were calculated using the microcomputer package PAUP (phylogenetic Analysis Using Parsimony, Version 2.4) developed by Safford (Illinois Natural History Survey, 607 East Peabody Drive, Champaign, Ill., USA) (Yang and Quiros, 1993).

RESULTS AND DISCUSSION

Effect of different doses of laser irradiation on leaf callus fresh weight (g/explants) of *Atropa belladonna*.

There was a significant difference between different doses of laser irradiation and callus fresh weight (g/explants). Data in Table (1) showed the effect of different doses of laser (0.0, 10, 15, 20, 25 and 30Jcm-$^{-2}$) on mutation of callus, as shown in Figure (1). The untreated callus with laser (control) and the callus treated with the level of laser 25Jcm^{-2} recorded the highest callus fresh weight (g/explants). Table (2) showed notable significant differences on callus fresh weight for all studied characters. The best dose was 25Jcm^{-2}. The mean values of leaf fresh weight of *Atropa belladonna* during first, second and third months after sowing were 5.174, 5.523 and 5.768g (at 25Jcm^{-2}), respectively, while these were 2.762, 2.467 and 2.379g (at 30Jcm^{-2}), respectively compared with the control values which were 1.885, 2.095 and 2.312g, respectively. These results are in harmony with Amiri *et al*. (2011) for MS with Kin in combination with 2, 4-D were suitable for maximal callus induction from leaf explants of *Datura stramonium* L. and *Withania somnifera* (Taha *et al*., 2008). The effect of different concentrations of 2, 4-D and BA or NAA and Kin added to MS on callus production from internode explants of *Catharanthus roseus*, *Celosia argentea*, and *Cordyline terminalis* was investigated. Medium supplemented with 1.0 mg/1 2,4-D + 3.0 mg/1 BA yielded the highest calli production value (Vasilevski *et al*.. 2001).

Table (1): Interaction between laser irradiation and hormones on leaf callus fresh weight induction *Atropa belladonna* plant.

	Callus leaf fresh weight							
First month	**Treatments**	**Control**	**10 J cm^{-2}**	**15 J cm^{-2}**	**20 J cm^{-2}**	**25 J cm^{-2}**	**30 J cm^{-2}**	**Means**
	1 mg/L (BAP) +1 mg/L (NAA)	1.738	2.023	2.640	4.480	5.055	2.753	3.115
	2 mg/L (2,4-D)+ 0.5 mg/L (Kin)	1.730	1.925	2.573	5.135	5.655	2.738	3.293
	3 mg/L (2,4-D)+0.5 mg/L (Kin)	2.188	2.178	2.228	4.260	4.813	2.795	3.077
	Means	**1.885**	**2.042**	**2.480**	**4.625**	**5.174**	**2.762**	**3.161**
		M	**NS**		**NS**			
		T	**0.661**		**0.880**			
		M x T	**NS**		**NS**			
Second months	**Treatments**	**Control**	**10 J cm^{-2}**	**15 J cm^{-2}**	**20 J cm^{-2}**	**25 J cm^{-2}**	**30 J cm^{-2}**	**Means**
	1 mg/L (BAP) +1 mg/L (NAA)	**1.810**	2.253	2.635	5.013	5.625	2.533	3.311
	2 mg/L (2,4-D)+ 0.5 mg/L (Kin)	**2.098**	2.343	2.740	5.505	5.840	2.650	3.529
	3 mg/L (2,4-D)+0.5 mg/L (Kin)	**2.378**	2.458	2.360	4.560	5.103	2.218	3.179
	Means	**2.095**	**2.351**	**2.578**	**5.026**	**5.523**	**2.467**	**3.340**
		M	**NS**		**NS**			
		T	**0.592**		**0.789**			
		M x T	**NS**		**NS**			
Third months	**Treatments**	**Control**	**10 J cm^{-2}**	**15 J cm^{-2}**	**20 J cm^{-2}**	**25 J cm^{-2}**	**30 J cm^{-2}**	**Mean**
	1 mg/L (BAP) +1 mg/L (NAA)	2.008	2.405	2.773	6.143	5.893	2.463	3.614
	2 mg/L (2,4-D)+ 0.5 mg/L (Kin)	2.358	3.265	2.838	5.753	6.058	2.485	3.793
	3 mg/L (2,4-D)+0.5 mg/L (Kin)	2.570	2.763	2.413	4.618	5.355	2.190	3.318
	Mean	**2.312**	**2.811**	**2.674**	**5.504**	**5.768**	**2.379**	**3.575**
		M	**NS**		**NS**			
		T	**0.659**		**0.877**			
		M x T	**NS**		**NS**			

At the 0.05 level, the mean difference is significant.

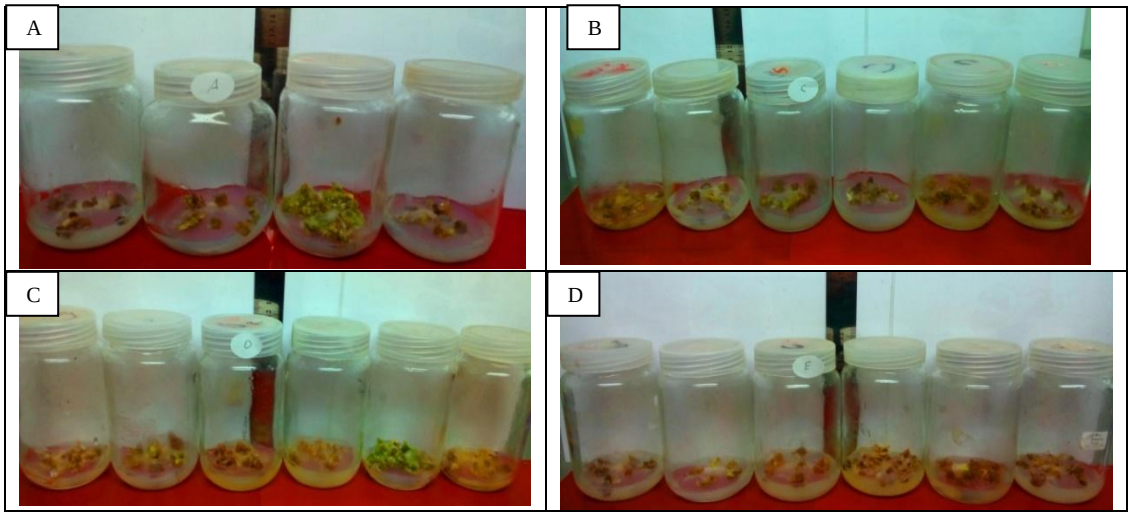

Fig. (1): Effect of laser irradiation and some growth regulators and the interaction between them on leaf callus fresh weight of *Atropa belladonna.* A: leaf media, B: Control + 1 mg/L (BAP) +1 mg/L (NAA), C:Control + 2 mg/L (2,4-D)+0.5 mg/L (Kin), D: Control + 3 mg/L (2,4-D)+0.5 mg/L (Kin).

Effect of different doses of laser irradiation on stem callus fresh weight (g/explants) of *Atropa belladonna*.

Data in Table (2) show the effect of different doses of laser irradiation (0.0, 10, 15, 20, 25 and 30 Jcm^{-2}) on mutation of callus as shown in Figure (2). The untreated callus with laser doses (control) and the callus treated with the dose ($25Jcm^{-2}$) recorded the highest fresh weight (g/explants).Table 2 showed notable significant differences on callus fresh weight for all studied characters. The best dose was $25Jcm^{-2}$. The mean values of fresh weight stem of *Atropa belladonna* L. during first, second and third months after sowing date were 5.415, 5.550 and 5.578 g (at $25Jcm^{-2}$), respectively, while these were 2.430, 2.295 and 2.235g (at $30Jcm^{-2}$) compared with the control which were 1.408, 1.713 and 1.780g, respectively. These results are in harmony with Atienzar *et al*., 2000 who found that laser treatments caused cell elongation, which increased gibberellic acid and increased cell vacuoles.

Table (2): Interaction between laser irradiation and hormones stem callus fresh weight of *Atropa belladonna*.

	Treatments	Control	10 J cm^{-2}	15 J cm^{-2}	20 J cm^{-2}	25 J cm^{-2}	30 J cm^{-2}	Mean
First month	2mg/L (BAP) +1 mg/L (NAA)	1.855	1.800	2.123	3.695	4.568	2.178	2.703
	1mg/L (2,4-D)+1 mg/L (BAP)	1.408	2.120	2.403	4.478	5.415	2.430	3.042
Second month	2mg/L (BAP) +1 mg/L (NAA)	2.010	2.095	2.210	4.315	5.103	2.060	2.965
	1mg/L (2,4-D)+1 mg/L (BAP)	1.713	2.285	2.558	5.055	5.550	2.295	3.243
Third month	2mg/L (BAP) +1 mg/L (NAA)	1.803	2.235	2.265	4.540	5.010	1.905	2.960
	1mg/L (2,4-D)+1 mg/L (BAP)	1.780	2.383	2.623	3.910	5.578	2.235	3.085

• At the 0.05 level, the mean difference is significant.

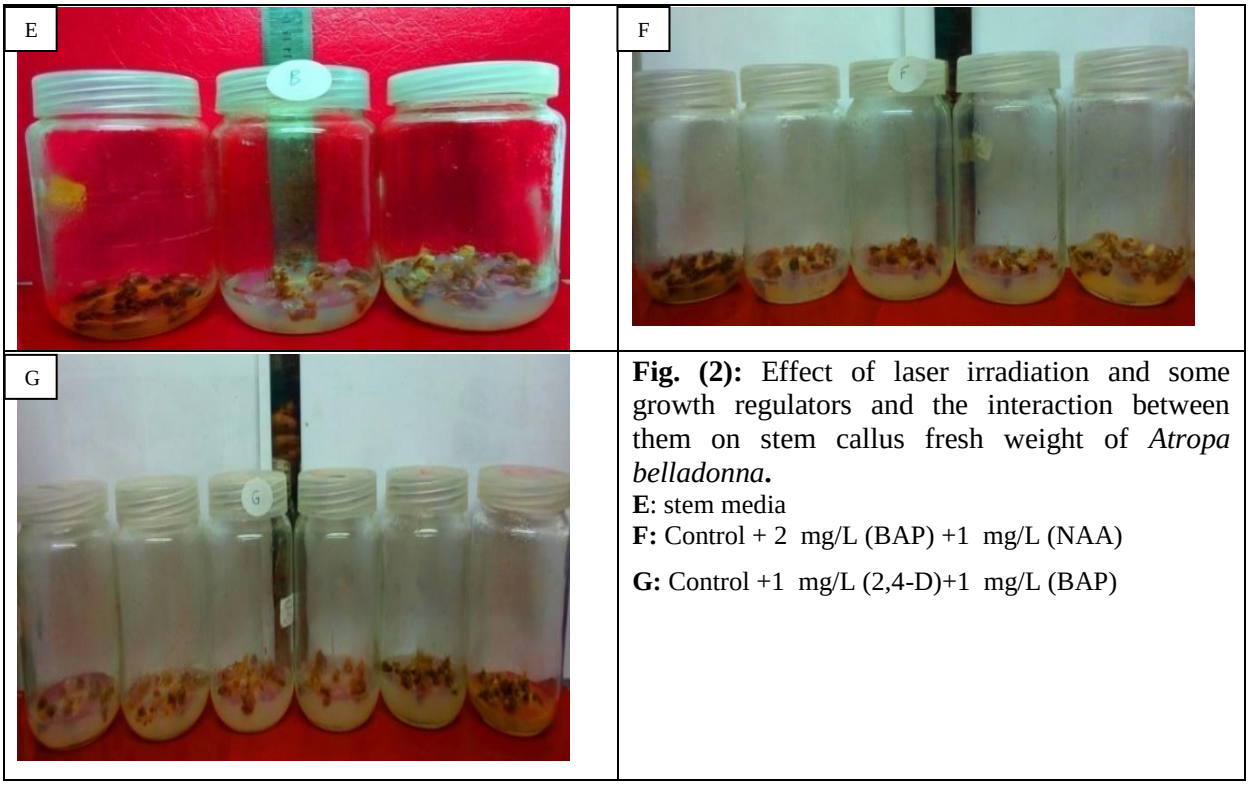

Fig. (2): Effect of laser irradiation and some growth regulators and the interaction between them on stem callus fresh weight of *Atropa belladonna*.
E: stem media
F: Control + 2 mg/L (BAP) +1 mg/L (NAA)
G: Control +1 mg/L (2,4-D)+1 mg/L (BAP)

Callus dry weight:

The treated callus with the different doses of laser irradiation (0.0, 10, 15, 20, 25 and 30Jcm^{-2}) showed that the best dose was 25Jcm^{-2}, which recorded the highest callus dry weight (g/explants) compared with (30Jcm^{-2}) and over the control. Table 3 showed notable significant differences on callus dry weight from leaf explants of *Atropa belladonna* L. after 4 weeks incubation periods for all studied characters. mutation of callus, as shown in Figure (3). The best dose was 25Jcm^{-2}. The mean values of callus which treated with hormones interaction for leaves dry weight of *Atropa belladonna* plant were 0.404 g. at 25Jcm^{-2}, compared with the control which was 0.215g. These results are in harmony with (Hamad and Jassem, 2011) who examined various explants (shoot tip, cotyledon and hypocotyls) from *Atropa belladonna* for callus production. Results showed that higher fresh and dry weights of callus were induced from shoot tip.

Table (3): Interaction between laser irradiation and hormones on dry weight of *Atropa belladonna* leaves.

Treatments	Control	10 J cm^{-2}	15 J cm^{-2}	20 J cm^{-2}	25 J cm^{-2}	30 J cm^{-2}	Mean
1 mg/L (BAP) +1 mg/L (NAA)	0.163	0.213	0.203	0.300	0.360	0.123	0.227
2 mg/L (2,4-D) +0.5 mg/L (Kin)	0.160	0.228	0.198	0.345	0.518	0.138	0.264
3 mg/L (2,4-D) +0.5 mg/L (Kin)	0.323	0.210	0.198	0.278	0.335	0.098	0.240
Mean	0.215	0.217	0.199	0.308	0.404	0.119	0.244
	M	NS		NS			
	T	0.068		0.090			
	M x T	0.117		0.156			
The mean difference is significant at the 0.05 level							

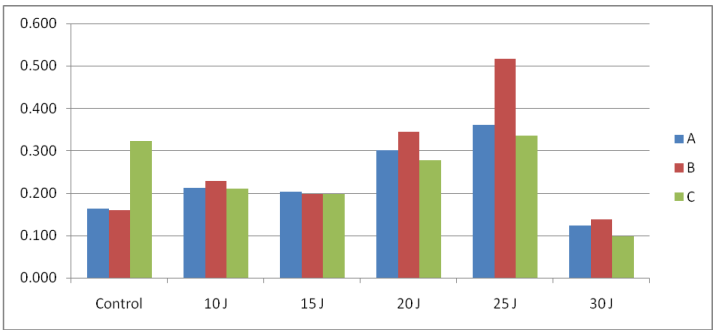

Fig. (3): Effect of laser irradiationon growth regulators and the interaction between them on callus dry weight of *Atropa belladonna* plant. A: hormone 1, B: hormone 2 and C: hormone 3.

Interaction between effect of laser irradiation and elicitor salicylic acid (SA) on leaves and stem callus fresh weight induction from *Atropa belladonna.*

Treatments are presented in Table (4) and Figure (4). With regard to the interaction between the effect of laser treatment and salicylic acid (SA) on leaves callus fresh weight, the highest significant value of callus fresh weight was recorded with laser doses +SA at 35 mg/l compared with the control. The best dose was 25 J cm^{-2} + 35 mg/l, after 4 weeks were 5.042g compared with the control which was 1.184g showed notable significant

differences on callus fresh weight for all studied characters. The best dose was 25 Jcm^{-2}.

Table (4): Interaction between effect of laser irradiation and elicitor salicylic acid (SA) on leaves callus fresh weight induction from *Atropa belladonna* plant.

	Callus fresh weight of Leaves	
Treatments	**First wieght**	**After 4 weeks**
Control	2.008	1.184
10 J cm^{-2}	3.29	1.356
15 J cm^{-2}	2.722	1.466
20 J cm^{-2}	5.542	4.168
25 J cm^{-2}	6.436	5.042
30 J cm^{-2}	2.218	1.232
Sig.	HS	HS
P-value	0.000	0.000
L.S.D 5%	0.57	0.35
L.S.D 1%	0.78	0.47

- The mean difference is significant at the 0.05 level. HS. =Highly significant

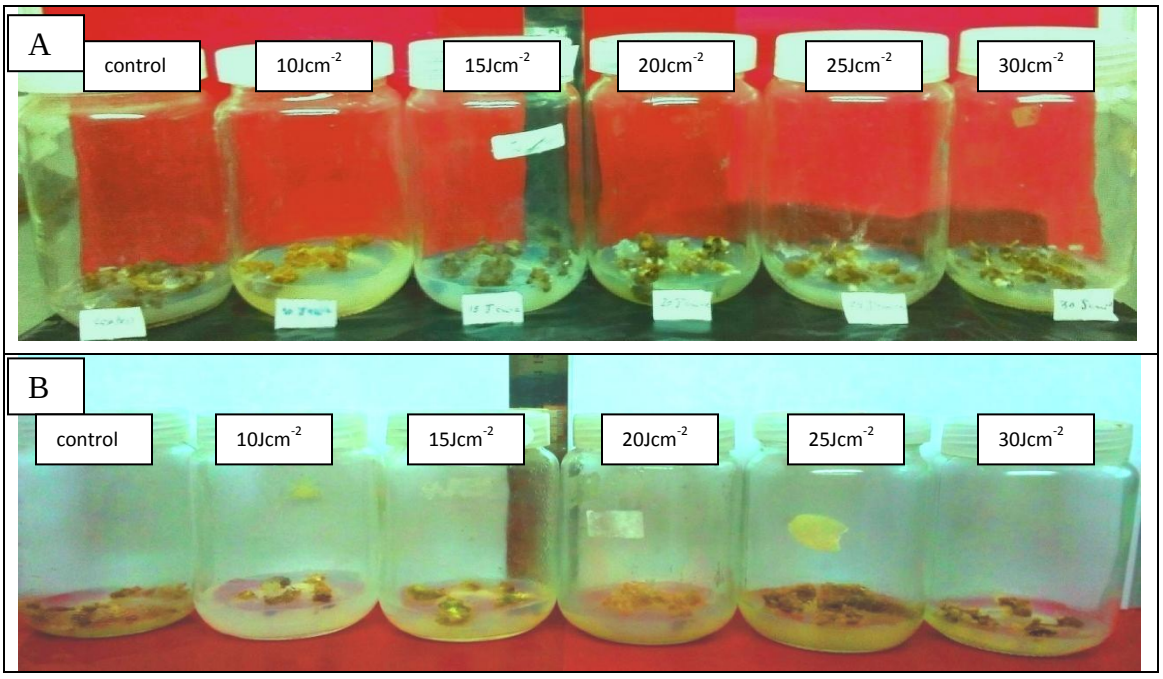

Fig. (4): Interaction between effect of laser irradiation, and elicitor salicylic acid on leaves callus fresh weight production *Atropa belladonna* Plant A: Control + 1 mg/L (2,4-D) +1 mg/L (BAP) + 35 mg/l SA. B: Control + 1 mg/L (2,4-D) +1 mg/L (BAP) + 35 mg/l SA after 4 weeks.

The mean values of leaves callus fresh weight of *Atropa belladonna* plant during first wieght after sowing date were 6.436 g (at 25 J cm^{-2}), while these were 2.218g (at 30 J cm^{-2}) compared with the control, which was 2.008 g, respectively. Similarly the results were recorded with SA at 35 mg/l only without laser effect showed

a negative effect on callus fresh weights comparing with control. In general SA has a negative effect on callus growth Chaichana and Dheeranupattana (2012).

Table (5):Effect of interaction between laser irradiation, and elicitor salicylic acid on stem callus fresh weight induction *Atropa belladonna* plant.

Treatments	Stem callus fresh weight	
	First weight	After 4 weeks
Control	1.932	1.048
10 J cm^{-2}	2.36	1.298
15 J cm^{-2}	2.446	1.4
20 J cm^{-2}	4.71	3.606
25 J cm^{-2}	5.438	4.898
30 J cm^{-2}	2.158	1.118
Sig.	HS	HS
P-value	0.000	0.000
L.S.D 5%	0.81	0.6
L.S.D 1%	1.09	0.82

The mean difference is significant at the 0.05 level. HS. =Highly significant.

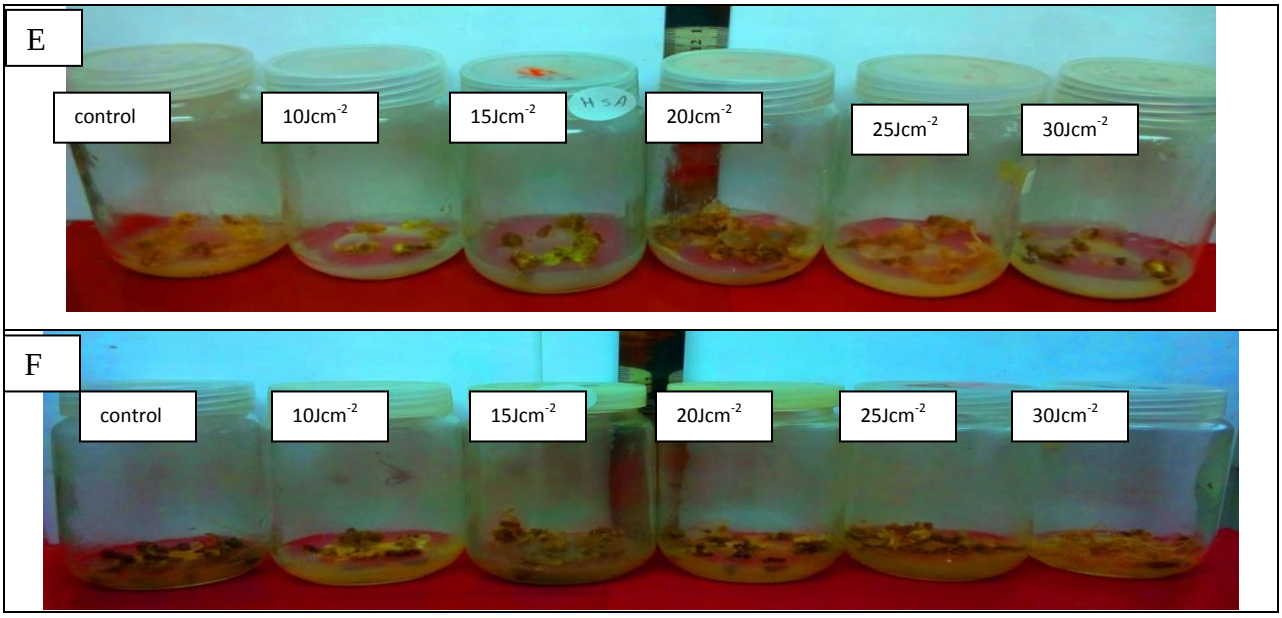

Fig. (5).Interaction between laser irradiation and elicitor salicylic acid on stem callus fresh weight of *Atropa belladonna* plant. E: Control + 2 mg/L (2,4-D) +0.5 mg/L (Kin) + 35 mg/l SA., F: Control + 2 mg/L (2,4-D) + 0.5 mg/L (Kin) + 35 mg/l SA after 4 weeks.

Treatments were presented in Table (5) and Figure (5) showed the effect of interaction between laser irradiation and salicylic acid (SA) on stem callus fresh weight, The highest significant value of stem callus fresh weight was recorded with laser doses + SA at 35 mg/l compared with the control. The best dose was 25 J cm^{-2} + 35 mg/l, after 4 weeks was 4.898g comparing with the control which was 1.932g. Significant differences on callus fresh weight for all studied characters were noticed; with the best dose was 25 Jcm^{-2}. The mean values of stem callus fresh weight, of *Atropa belladonna*

L. plant during first wieght after sowing date was 6.436 g (at 25 J cm^{-2}), while was 1.118g. (at 30 Jcm^{-2}) compared with the control which was 1.048 g, respectively.

Molecular genetic studies by ISSR experiments:
ISSR- molecular analysis of treatments callus by laser irradiation:

The characteristics of fragment primers summarized in table (6) and Fig. 6 the following are the amplification results of the *Atropa belladonna* obtained by the examined twelve ISSR primers(ISSR-1, ISSR-2, ISSR-3, ISSR-4, ISSR-6, ISSR-7, ISSR-8, ISSR-9, ISSR-10, ISSR-11, ISSR -12 and ISSR-13)Produced 154 bands within DNA template representing callus (Control) 10 J cm^{-2}, 15 J cm^{-2}, 20 J cm^{-2}, 25 J cm^{-2} and 30 J cm^{-2} callus treated different doses of *Atropa belladonna* was treated by laser irradiation. All bands produced by the twelve ISSR primers were monomorphic, while the primer ISSR-1primer revealed 13 bands divided into 6 monomorphic and 3 unique bands percentage of polymorphism (54%), while the ISSR-2 primer revealed 13 bands divided into 5 monomorphic and 3 unique bands percentage of polymorphism (62%). The ISSR-3 primer revealed 14 bands divided into 6 monomorphic and 1 unique bands Percentage of polymorphism (57%), while the ISSR-4 primer revealed 14 bands divided into 8 monomorphic and 2 unique bands percentage of polymorphism (43%). The ISSR-6 primer revealed 14 bands divided into 2 monomorphic and 2 unique bands, percentage of polymorphism (86%), while the ISSR-7 primer revealed 12 bands divided into 5 monomorphic and 2 unique bands percentage of polymorphism (58%), while the ISSR-8 primer revealed 14 bands divided into 5 monomorphic and 4 unique bands Percentage of polymorphism (64%).

The ISSR primer are annealed to homologous genomic microsatellite sequences (Fang and Roose, 1997) and allows amplification of regions located between two closely spaced, oppositely oriented microsatellites, with a reproducibility level of more than 99 % after performing repeatability tests for ISSR markers using DNA samples of the same cultivar grown in different locations, DNA extracted from different aged leaves of the same individual, and by performing separate PCR runs. As a result, ISSR and PCR produce a reproducible pattern of genomic fragments similar to a RAPD pattern but with more (up to 97) bands. Mahdy *et al*. (2021) used SSR for deferent between *Vigna* spp.

Table (6): Total number of ISSR fragments (N), number of monomorphic Unique, (P) and percentage (P%) of polymorphic fragments generated by primer.

Primer name	Primer sequence 5'-3'	Total No. of Bands	No. of Mono-morphic Bands	No. of Unique Bands	No. of Poly-morphic bands	Poly-morphism (P) %	Mean of Band frequency
ISSR- 1	5'-AGAGAGAGAGAGAGAGYC-3'	13	6	3	4	54	0.7
ISSR- 2	5'-AGAGAGAGAGAGAGAGYG-3'	13	5	3	8	62	0.6
ISSR- 3	5'-ACACACACACACACACYT-3'	14	6	1	8	57	0.7
ISSR- 4	5'-ACACACACACACACACYG-3'	14	8	2	4	43	0.8
ISSR- 6	5'-CGCGATAGATAGATAGATA-3'	14	2	2	10	86	0.5
ISSR- 7	5'-GACGATAGATAGATAGATA-3'	12	5	2	5	58	0.7
ISSR- 8	5'-AGACAGACAGACAGACGC-3'	14	5	4	9	64	0.6
ISSR- 9	5'-GATAGATAGATAGATAGC-3'	17	5	2	12	71	0.6
ISSR- 10	5'-GACAGACAGACAGACAAT-3'	12	5	3	4	58	0.7
ISSR- 11	5'-ACACACACACACACACYA-3'	12	5	4	8	71	0.6
ISSR- 12	5'-ACACACACACACACACYC-3'	10	6	0	4	40	0.7
ISSR- 13	5'-AGAGAGAGAGAGAGAGYT-3'	9	5	1	3	44	0.7
Total		154	63	27	79	70.80	

Percentage (%) of polymorphism = (No. of polymorphic (unique) bands ÷ Total bands) X 100.

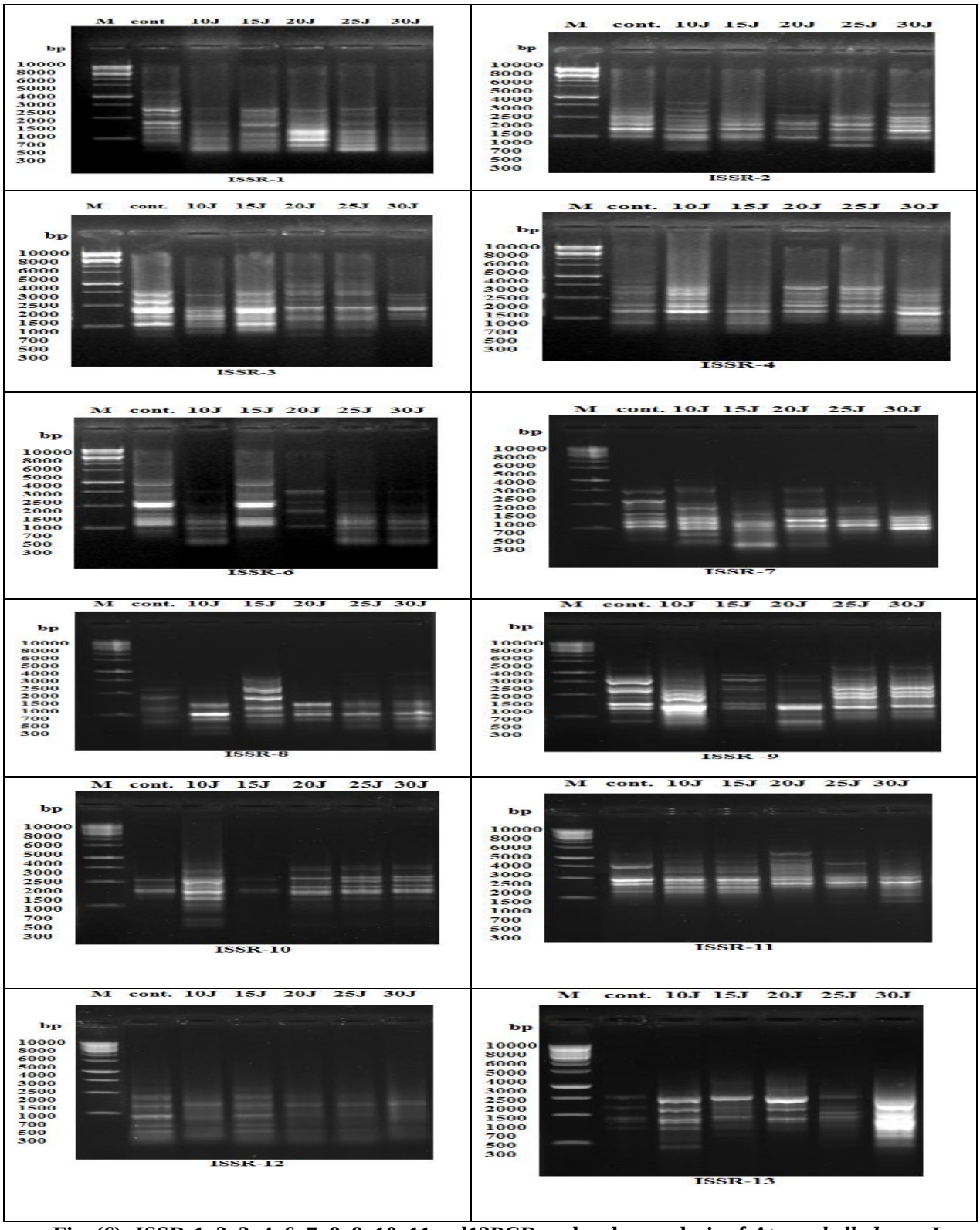

Fig. (6): ISSR-1, 2, 3, 4, 6, 7, 8, 9, 10, 11and12PCR molecular analysis of *Atropa belladonna* L. treatment by laser irradiation.

Cluster analysis (similarity index) based on ISSR-PCR analysis using UPGMA computer analysis was shown in Table (7). The highest similarity index recorded was 0.85, which was observed between the two treatments 25Jcm^{-2} and 30 Jcm^{-2}, while the lowest similarity index recorded was 0.74, which was observed between control and 30 Jcm^{-2}.

Table (7): Similarity index (Pair wise comparison) among five treatments usinglaser irradiationon ISSR-PCR analysis.

	Control	10 Jcm^{-2}	15 Jcm^{-2}	20 Jcm^{-2}	25 Jcm^{-2}	30 Jcm^{-2}
Control	100					
10 Jcm^{-2}	74	100				
10 Jcm^{-2}	84	76	100			
20 Jcm^{-2}	76	77	76	100		
25 Jcm^{-2}	73	79	73	79	100	
30 Jcm^{-2}	74	82	75	75	85	100

The genetic relationships among the treatment and wild type were represented by a dendrogram, as shown in Figure (7). The six treatments control, 10 J cm^{-2}, 15 J cm^{-2}, 20 J cm^{-2} 25 J cm^{-2} and 30 J cm^{2} *Atropa belladonna* were separated into tow clusters; cluster one included 20 J cm^{-2}, cluster 2 included 15 J cm^{-2} and control. Within cluster 1, three sub cluster contained two treatments 25 J cm^{-2} and 30 J cm^{-2}, while the second sub cluster contained 10 J cm^{-2}. Behera *et al.* (2006) used microsatellite markers and found greater diversity in 92 South Asian *Solanum melongena* accessions (genetic similarity between 0.37 and 0.90). According to the findings of this study, ISSR primers are effective in detecting polymorphism in Indonesian eggplant accessions, with 64.5 % polymorphic bands demonstrating eggplant genetic diversity.

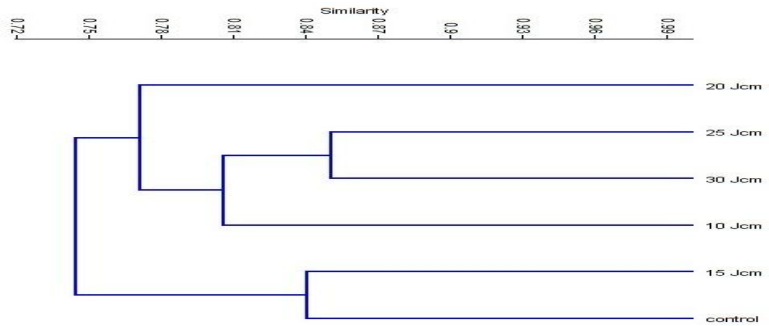

Fig. (7). Dendrogram for the genetic distances between five treatments using laser irradiation on ISSR-PCR analysis.

Effect of laser irradiation on secondary metabolites of *Atropa belladonna* callus.

HPLC of *in vitro* Atropine, Hyoscyamine and scopolamine from *Atropa belladonna.* The results of treatments were presented in Table (8) and Figure (8) showed there was significant difference on average of secondary metabolites of *Atropa belladonna* callus under the effect of different exposure doses of He–Ne laser. The best dose was 25 Jcm^{-2}and the mean values of secondary metabolite Atropine,

Hyoscyamine, and Scopolamine were analyzed using HPLC. accumulation of all alkaloids the content of atropine, hyoscyamine and Scopolamine were increased by 267.82(µg/m), 156.94 (µg/m) and 131.19 (µg/m) respectively, in comparison with the control which were 99.97 (µg/m), 44.13 (µg/m) and 36.75 (µg/m), respectively.

Table (8): HPLC analysis of atropine, hyoscyamine and scopolamine under the effect of interaction between laser irradiation and salicylic acid in callus of *Atropa* plant (% dry weight).

Treatments	Atropine (µg/m)	Hyoscyamine (µg/m)	Scopolamine (µg/m)
Control	99.97	44.13	36.75
$10Jcm^{-2}$	145.33	71.90	42.30
$15Jcm^{-2}$	198.46	96.13	92.44
$20Jcm^{-2}$	236.55	130.38	113.26
$25Jcm^{-2}$	267.82	156.94	131.19
$30Jcm^{-2}$	57.19	33.94	30.17
Control+ 35mg/l SA	132.66	87.18	52.13
$10Jcm^{-2}$+35mg/l SA	163.82	97.30	78.24
$15Jcm^{-2}$+35mg/l SA	214.22	132.75	129.95
$20Jcm^{-2}$+ 35mg/l SA	276.12	178.86	158.71
$25Jcm^{-2}$+ 35mg/l SA	280.76	203.77	189.03
$30Jcm^{-2}$+35mg/l SA	74.10	58.97	47.37

There have been many attempts to enhance and improve the production of tropane alkaloids by transgenic cultures (Yang et al. 2011). The use of gamma radiation and gibberellic acid for the production of mutant plant seeming to achieve high alkaloid content is also reported by (Khater *et al*., 2013).

Effect of interaction between laser irradiation and elicitor salicylic acid (SA) on callus active ingredient contents atropine, hyoscyamine and scopolamine from *Atropa belladonna*.

Elicitation is the process of inducing or enhancing secondary metabolite synthesis by plants in order to ensure their survival, persistence, and competitiveness, as well as discussing the classification of elicitors, their mechanism of action, and applications for the production of phytopharmaceuticals from medicinal plants. Figure (9) show that there were significant differences on average of secondary metabolites of *Atropa belladonna* L. callus under the effect of different exposure doses of laser irradiation and elicitor salicylic acid SA on secondary metabolites of *Atropa belladonna* callus. The best dose was 25 Jcm^{-2}+35 mg/l SA. The plant material was dried at a temperature not exceeding 40°C and ground analyzed using HPLC. The highest dry weight was recorded with the callus on MS medium supplemented with SA at 35mg/l after 30 days. Accumulation of both alkaloids atropine and scopolamine in callus were enhanced increased by 280.76 (µg/m), 203.77 (µg/m) and 189.03 (µg/m), respectively in comparison the callus control 132.66 (µg/m), 87.18(µg/m) and 52.13 (µg/m), respectively. Stress causes secondary metabolites to accumulate in the plant body (Fig. 10). Elicitor is a type of stress agent that increases the production of secondary metabolites in a specific tissue, organ, or cell. The use of elicitors in plant tissue culture has recently opened up a new avenue for the production of secondary metabolite compounds (Naik and Al-Khayri 2016; Abd-Rahman, 2008).

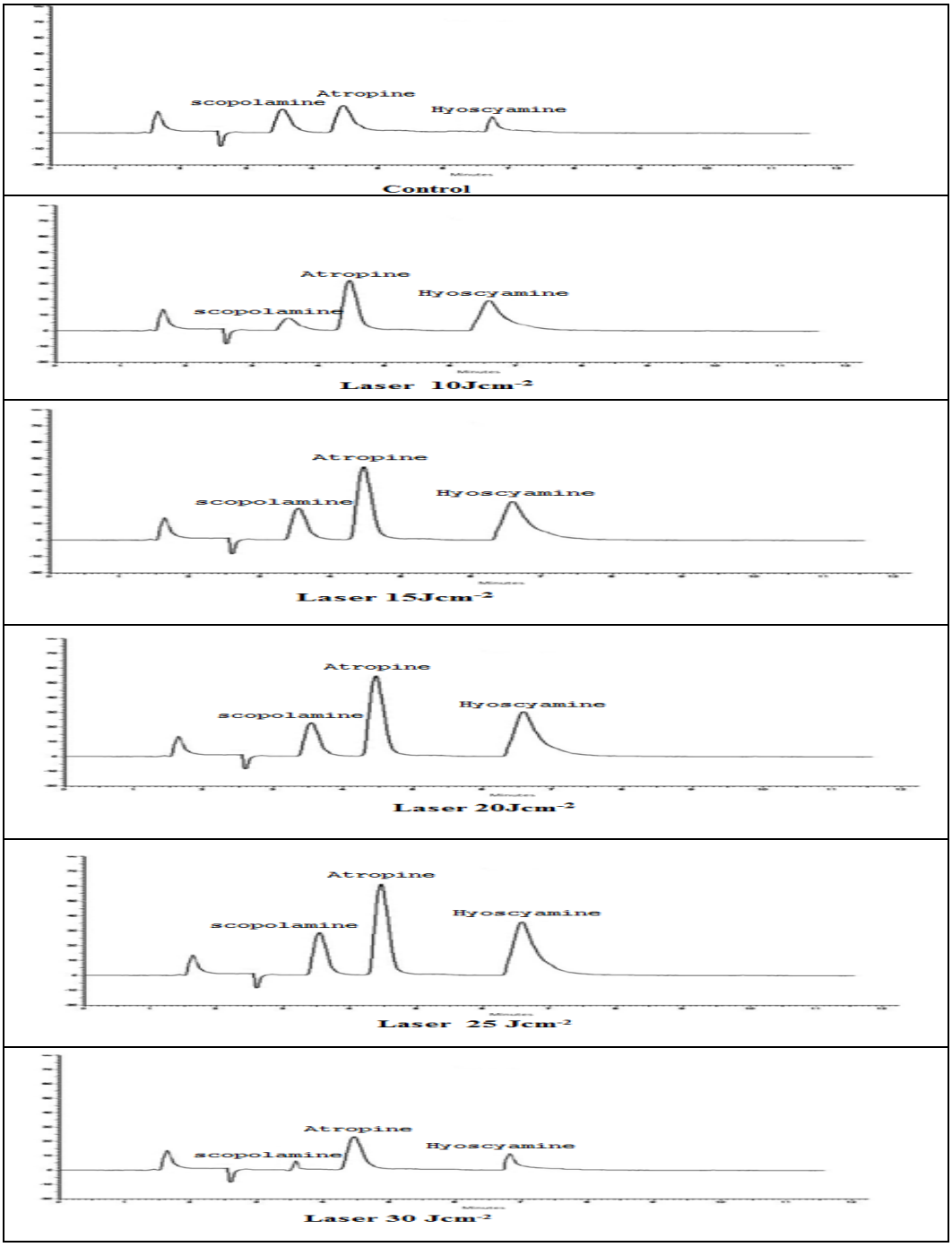

Fig. (9): Effect of different exposure doses of laser irradiation secondary metabolites of *Atropa belladonna* callus.

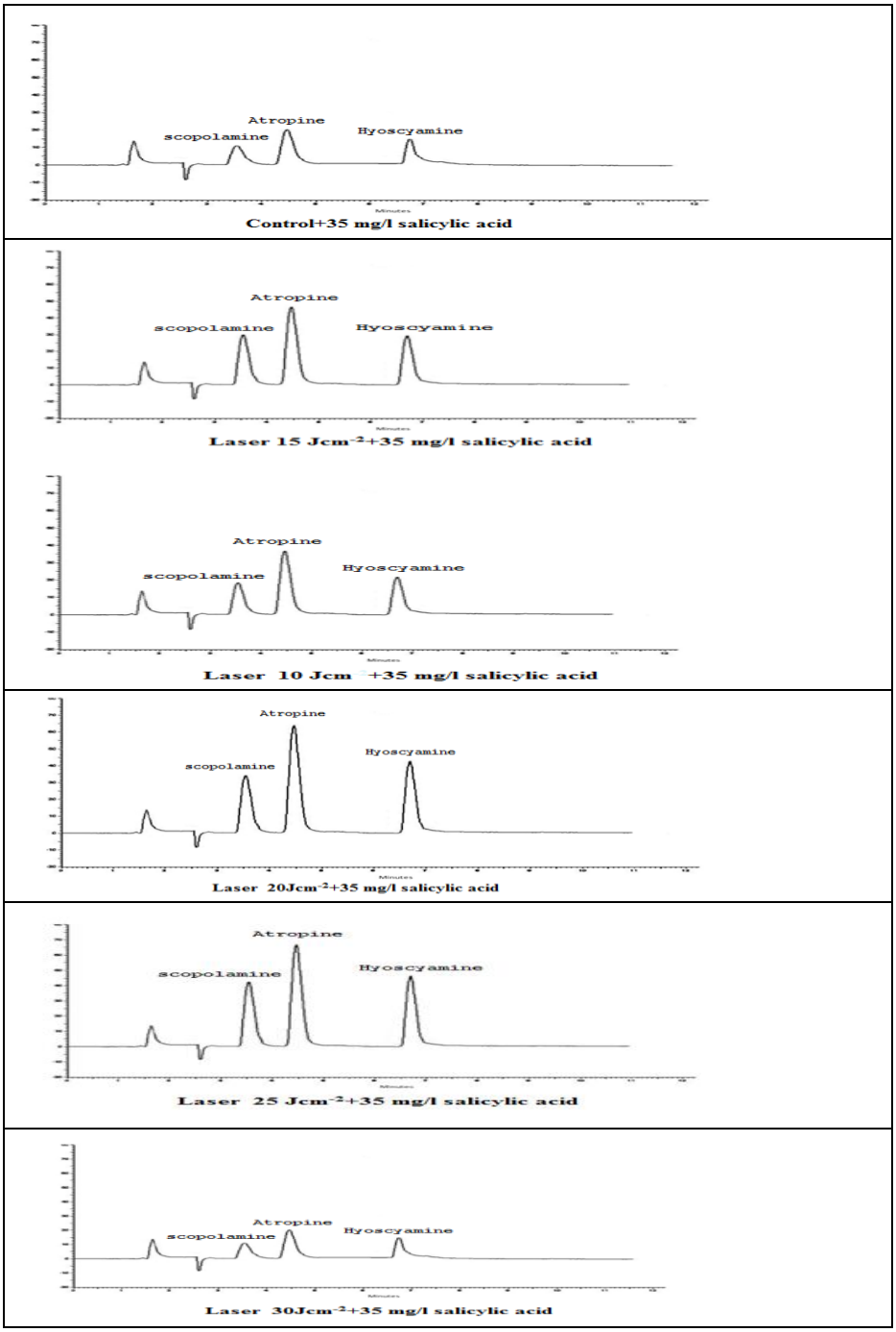

Fig. (10):Interaction between the effect of He–Ne laser irradiation and elicitor salicylic acid (SA) on secondary metabolites of *Atropa belladonna* callus.

Conclusions

In comparison to the control, He– Ne laser treatment of callus increased, particularly with laser treatments (25 Jcm^{-2}). With leaf explants cultured on MS medium supplemented with2 mg/L (2,4-D) + 0.5 mg/L (Kin) +35 mg/l elicitor salicylic acid (SA), the highest values of fresh and dry

weights of the formed callus were obtained. The results of *in vitro* High Performance (Pressure) Liquid Chromatography (HPLC) analysis revealed significant differences on the treated callus of leaf and stem for all studied traits. Laser treatments have a significant impact on gene expression by turning genes on and off. ISSR analysis and other growth parameters are regarded as critical tools for detecting DNA profile changes caused by laser treatments.

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دراسات وراثية جزيئية على *Atropa belladonna* L. تحت تأثير إشعاع الليزر وحمض الساليسيليك على الأتروبين والسكوبولامين والهيوسيامين بتقنيات زراعة الأنسجة

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المستخلص

Atropa belladonna هي أهم مصدر تجاري لقلويدات الأتروبان الصيدلانية مثل الأتروبين ، سكوبولامين ، والهيوسيامين. تهدف الدراسة الحالية إلى التحقق من تأثير 6 جرعات مختلفة من ليزر الهليوم-نيون (0.0 ، 10 ، 15 ، 20 ، 25 ، 30 جول سم $^{-2}$) على الوزن الطازج للكالس وتطبيق أدوات التكنولوجيا الحيوية كنهج بديل لزيادة تراكم ال مواد الفعالة (الأتروبين) لنبلت البيلادونا بـإستخدام مادة غير حيوية للتغذية في البيئة مثل حمض الساليسيليك. تم اجراء دراسات علي المستوي الجزيئي بإستخدام إثني عشر بادئات ISSR تحت ستة جرعات إشعاع ليزر . كذالك تم إستخدام تقنية HPLC لتحديد محتويات الأتروبين والسكوبولامين والهيوسيامين. أظهرت النتائج أن أعلى وزن للكالس الطازج ، الوزن الجاف للكالس تم الحصول علية من الكالس الفاتح من (الأوراق والساق) والتي تم زراعتها علي وسط (MS) مضاف اليها تركيزات مختلفة من منظمات النمو مثل NAA، 2،4 D - و Kin و BAP (عند 0.5 و 1.0 مجم / لتر). من ناحية أخرى ، أثرت جميع معاملات الليزر علي أنسجة الكالس والمكونات النشطة طبيًا من *Atropa belladonna*. تقترح هذه الدراسة طريقة أكثر كفاءة للإنتاج التجاري واسع النطاق للأتروبين ، والسكوبولامين ، والهيوسيامين بواسطة نباتات *A. belladonna*.