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Research Article

TROPANE ALKALOIDS PRODUCTION FROM CALLUS CULTURE OF *ATROPA BELLADONNA* L. AS AFFECTED BY ELICITORS AND PRECURSOR FEEDING

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ABSTRACT

Atropa belladonna L is most important commercial source of pharmaceutical tropane alkaloids. Initiation of callus culture on MS solid media with different concentrations of growth regulators as BA and 2,4 D from different explants shows that 2,4-D only at 1.0mg/L and 2.0mg/l gave the highest callus formation score after 21 days. The highest concentration of atropine (376.62 µg/g DW) and scopolamine (103.16 µg/g DW) were obtained from leaf callus on MS medium supplemented with 2,4 D at 0.5 mg/L after 28 days. The effect of elicitors and precursor feeding on tropane alkaloids production in callus culture were examined. Accumulation of both alkaloids; atropine and scopolamine in callus were enhanced after 8 days with jasmonic acid at concentrations (50 µM), after 15 days with yeast extract at concentrations (0.5 g/L) and after 21days with ornithine at 1mM in comparison with control callus. Salicylic acid inhibits callus growth and accumulation of atropine and scopolamine in treated callus.

Keywords: *Atropa belladonna* L, callus, growth regulators, elicitors, ornithine, tropane alkaloids

INTRODUCTION

The genus *Atropa* belongs to family Solanaceae, tribe Hyoscyameae, and consists of 4 species, *Atrop aacuminata* Royle, *Atropa belladonna* L., *Atropa baetica* Willk., *Atropa pallidiflora* Schönb.-Tem., which are distributed in the Mediterranean region, South Europe and Asia [1].

Atropa belladonna commonly known as belladonna or deadly nightshade is a perennial herbaceous plant and most important commercial source of pharmaceutical tropane alkaloids in the family of Solanaceae native to Europe, North Africa and Western Asia[2]. Tropane alkaloids are a group of secondary metabolites containingan 8-azabicyclo[3.2.1]octane nucleus skeleton as a key structural element[3]. They have been identified not only in Solanaceae and Erythroxylaceae, where they are the most abundant alkaloids but also in other families, e. g. Proteaceae, Euphorbiaceae, Rhizophoraceae, Convolvulaceae, Cruciferae and Moraceae[4].

The active agents present in *belladonna* include atropine and scopolamine has anticholinergic effects on central and peripheral nervous system[5–7] so it is used for control some symptoms of parkinson's disease and serves as an antidote for poisoning by organophosphate insecticides and nerve gases [6-9].

Atropine is used as mydriatic, antispasmodic and antihyperhidrosis[6]. Scopolamine is used as an antiemetic against motion sickness or for people suffering from nausea as a result of receiving chemotherapy[10].

In October 2006 researchers at US National Institute of Mental Health found that scopolamine reduces symptoms of depression within a few days and the improvement lasted for at least a week after switching to placebo[11].

These alkaloids cannot be substituted by any other class of compounds, so they are still in demand. This is one of the reasons for the development of an active field of research into the metabolism of the alkaloids, the enzymes involved, and the genes that produce them[12].

Recent advances in the field of plant biotechnology show the potential of using plant cell and tissue cultures as a source for the large-scale production of valuable secondary metabolites instead of using whole plants and subsequent extensive land exploitation. Moreover, the employment of molecular biology techniques has allowed for obtaining novel products from genetically engineered plants[13]. Exposure of cell or hairy root cultures to a variety of biotic (as salicylic acid, methyl jasmonate and yeast extract) and abiotic (inorganic salts and UV irradiation,) factors have been shown to improve the yield of secondary metabolites [14].

MATERIALS AND METHODS

Seed Sterilization and Germination

Atropa belladonna L seeds were washed with tap water, immersed in clorox 50% v/v (2.5% sodium hypochlorite) for 20 min and then rinsed with sterile distilled water under sterile conditions in a laminar airflow cabinet till free from chloride. The seeds were aseptically cultured in glass jars containing MS (Murashige & shook, 1962)[15] medium, which contained 30 g/L sucrose and 8g/L agar. The culture media was adjusted to pH 5.8 and then sterilized by autoclaving at 121°C and 15 pound/inch^2 over 20 minutes. Seed germination occurred after 10 days, transferred to another MS medium, then one node stem segments

of 4 weeks old seedlings were used as explants for the multiplication. Cultures were maintained at 25 ± 2°C and 16/8 h photoperiod and sub cultured every month.

Effect of Kinetin (Kin) and Benzyladenine (BA) on Multiplication of *Atropa belladonna* L. In vitro Culture

In this experiment, several MS media were supplemented separately with BA and Kin at concentration (0, 0.5,1 and 2 mg\L). One node stem segments of 4 weeks old invitro seedlings were used as explants for the multiplication. Each treatment consisted of three replicates (each jar contained four explants). Data were recorded after 8 weeks for number of branches and length (cm) of shoot, and root.

Effect of 2,4 Di-Chlorophenoxyacetic Acid (2,4 D) and BA on Callus Induction

In this experiment, Sterile explants (leaves, shoots, roots) from invitro were cultured on solid MS medium supplemented with different concentrations of 2,4 D and BA in combined form (0.0, 0.5, 1and 2 mg/L) to study their effects on callus induction. Each treatment consisted of three replicates (each jar contained four explants). Cultures were maintained at 25 ± 2°C in dark. Callus responses were estimated after 21days. The amount of callus was estimated by a '0-4 Scale' scoring system [16] Table 1.

Effect of 2,4 D on Tropane Alkaloids Production from Callus Culture from Different Explants

In this experiment, Sterile explants (leaves, shoots, roots) from invitro were cultured on MS medium supplemented with different concentrations of 2,4-D (0.25, 0.5, 1and 2 mg/L) to study their effects on tropane alkaloids production from callus culture. Each treatment consisted of three replicates (each jar contained four explants). Cultures were maintained at 25 ± 2°C in the dark. Callus initiated from each treatment after one month was transferred to fresh media of the same composition and after another one-month callus was subcultured on 2,4-D free medium for month more, then callus was harvested for determination of dry weight and tropane alkaloids concentration after extraction.

Elicitors and Precursor Feeding of Callus Culture

Elicitation was performed by jasmonic acids, yeast extract and salicylic acid.Each elicitor was added to MS media in the following concentrations[17]:

a) Jasmonic acids: 0, 10, 25, 50 µM.
b) Yeast extract: 0, 0.5, 1 and 2 g/L.
c) Salicylic acid: 0, 0.5, 1 and 2 mM.
d) Ornithine as amino acid precursor: 0,1, 2 and 3 mM[18].

Each treatment consisted of three replicates. Cultures were maintained at 25± 2°C in the dark. The samples were taken after 8,15 and 21 days from cultivation.

Determination of Dry Weight and Total Alkaloid

Samples were collected and washed to remove remaining media and prepared to measure fresh weight then dried in oven at 60 °C to measure dry weight. Callus (100 mg) was powdered and then sonicated for 30 min with 10 ml of methylene chloride-methanol-ammonium hydroxide (25%) (15:15:1), left at room temperature for 1 hr then filtered and washed with 1 ml of methylene chloride twice. After solvent evaporation, 5 ml of methylene chloride and 2 ml of sulfuric acid (1N) were added to the dried residue and mixed thoroughly, then methylene chloride layer was removed, and the acidic layer was adjusted to pH 10 by using NH_4OH. The alkaloids were extracted by methylene chloride (2 ml) 3 times. The combined extract was filtered and $Na_2SO_4$was washed with 1 to 2 ml of methylene chloride, then evaporated and the residues were separately dissolved in 1 ml methanol and kept at -20°C until analysis[19]. HPLC analysis was carried out on a Agilent HPLC system, equipped with a Inertsil ODS-3 column (5 µm, 250 x 4.6 mm) and a UV detector. The samples were analyzed using 50 mM potassium dihydrogen orthophosphoric acid buffer (adjusted to pH 3.0 by orthophosphoric acid): Acetonitrile (80:20 v/v). The flow rate was 1 ml /min and detection was carried out at a wavelength of 215 nm[19]. Calibration curves of atropine and scopolamine standard were constructed by their concentration against peak area. The results are illustrated in Table 2 and Figures 1a & 1b.

Statistical Analysis

Data of elicitation and precursor feeding experiments were statistically analyzed as a factorial experiment. Randomized Complete Block Design were used to find the analysis of variance (ANOVA). Comparisons among means were made via the least significant differences multiple range tests according to Snedecor (1989).[20] The data were analyzed using MSTATC software program. The experiments were performed in triplicate and the mean values were given.

RESULTS AND DISCUSSION

Effect of Kin and BA on Multiplication of *Atropa belladonna* L. In vitro Culture

Table 3 shows effect of different concentrations of growth regulators BA and Kin (0, 0.25, 0.5, 1and 2 mg/L) on multiplication of *Atropa belladonna* L. Branches number did not affected significantly by Kin while increase with high BA concentration where the highest mean number of branches (16.5 branches/explant) was recorded when the explants were cultured on MS medium supplemented with BA 2mg/L. In contrast, shoot length and root length were inhibited by high concentration of BA and Kin. The highest mean shoot length (15.5 cm) obtained by Kin at 0.25 mg/L and control medium and the highest mean root length (12 cm &11.75cm) obtained by 0.5 mg/L Kin and control medium respectively. Reddy et al.(2011) [21] reported that the mean of shoots lengths decreases by increasing the concentration of BA in MS medium. Li et al. (2006) and Yuniastutiet al. (2016) [22,23] reported that cytokines (BA & Kin) inhibits formation of lateral roots and elongation of primary roots.

Effect of 2,4 D and BA on Callus Induction

The effect of BA and 2,4-D in different concentrations on callus formation, from different explants (leaf, stem and root) after 21days was studied using a '0-4 Scale' scoring system. Data are shown in Table 4, indicated that the effect of MS medium contained 2,4-D only at 1 mg/L and 2 mg/L gave the highest callus formation score. No callus was observed with MS medium free from growth regulators specially with 2,4-D. BA had no effect at any used concentration. Khater *et al.(*2013) [24] reported that there were a markedly increases in callus induction percentage of *Atropa belladonna* L by increasing 2, 4-D concentrations until 2 mg/L. Highly significant differences between two concentrations of 2, 4-D (1 and 2 mg/L) were recorded on callus fresh weight and callus dry weight respectively. The same findings were reported by Castellar *et al. (*2011) decided that efficient callus induction from leaf explants of *Petiveria alliacea* L was observed on media supplemented with 2, 4-D[25]. The best results for callus formation in *Bacopa*

monnieri(L.) Penn. was obtained in the leaf explants on MS supplemented with 0.5 mg/L 2, 4 -D[26]. Showkat*et al.* (2010) found that media contained 3 mg/L of 2,4-D was best for the induction of Sugarcane callus with 100% callus induction rate. This response is the same with many other plants because 2,4-D is the primary auxin which is used for the callus induction. The medium contained 2,4-D in the concentration of 4 mg/L also produced the same result but with much less regeneration potency. The higher amount of this auxin may result in the loss of the regeneration potential of the callus[27].

Effect of 2,4 D on Tropane Alkaloids Production from Callus Culture from Different Explants

Figure 2 and Table 5 shows results of using different concentrations (0, 0.25, 0.5, 1 and 2 mg/L) of 2,4 D on dry weight and tropane alkaloids production from callus culture from different explants of *Atropa belladonna* L after one month. The highest mean dry weight (1.137 g and 1.057g) were showed with stem callus on MS medium supplemented with 2,4 D at 0.25 mg/L and leaf callus on MS medium supplemented with 2,4 D at 1.0 mg/L respectively. The highest concentration of atropine (376.62 µg/g DW) and Scopolamine (103.16 µg/g DW) were obtained from leaf callus on MS medium supplemented with 2,4 D at 0.5 mg/L. Our results show that differentiation and alkaloidal contents of callus cultures form different explants decrease with increase 2,4 D concentration as illustrated in Figure 3.

Palazón et.al (1995) [28] reported that the addition of 1.0 µM 2,4-D to the culture medium had a positive effect on callus biomass production, while it inhibited root formation by this tissue (the lower the 2,4-D concentration in the medium the greater the number of roots which emerged from the calli) of Datura species. The auxin concentration in the culture medium controls the production of nicotine. The calli grown at lower auxin concentrations had significantly higher levels of nicotine than those grown at higher auxin concentrations[29–31]. In comparison with 0.1 µM 2,4-D, the 1 µM 2,4-D inhibited to a larger degree organogenesis and the ability of transformed calli to biosynthesize tropane alkaloids;[32] this result agrees with those of Robins et al. (1991) [33], which prove that high concentrations of 2,4-D added to D. stramonium transformed roots caused the disorganisation of the roots and hyoscyamine biosynthesis inhibition.

Elicitors and Precursor Feeding

Effect of Jasmonic Acid (JA)

Table 6 shows results of using different concentrations (0, 10, 25 and 50 µM) of JA on dry weight and tropane alkaloids production from leaf callus of *Atropa belladonna* L for 21 days. No significant difference between treated callus and control callus in dry weight. Accumulation of both alkaloids atropine and scopolamine in callus were enhanced after 8 days with JA at concentrations (50 µM) where the content of atropine and scopolamine was increased 479.3% and 70.4 % respectively, in comparison with control callus. After 15 days alkaloidal contents in treated callus increase with time but lower than control as illustrated in Figure 4. The jasmonate was found to be inhibitors for callus growth and somatic embryogenesis in *Medicago sativa* L. tissue cultures[34].

Effect of Yeast Extract (YE)

Table 7 shows results of using different concentrations (0, 0.5, 1 and 2 g/L) of YE on dry weight and tropane alkaloids production from leaf callus of *Atropa belladonna* L for 21 days. The highest dry weight was recorded with a callus on MS medium supplemented with YE at 1 g/L after 15 days. Accumulation of both alkaloids atropine and scopolamine in callus were enhanced after 8 day and the highest alkaloidal contents were recorded after 15 days with YE at concentrations (0.5 g/L) where the content of atropine and scopolamine was increased by 32.2% and 2.2% respectively, in comparison with control callus. After 21 days alkaloidal contents in treated callus decrease with time as illustrated in Figure 5. In study on callus cultures of *Hyoscyamus muticus* L, YE at low and moderate levels (0.25 and 0.5 g/L) caused the same value of callus dry weight (0.15 g/explant), also (0.75 and 1.0 g/L levels) formed the same value with increasing of callus dry weight (0.18 g/explant), while gave fluctuating concentration of hyoscyamine as follows 1.73, 1.20, 1.57 and 1.17 mg/g dry weigh, respectively[35]. In another study on *Zingiber officinale* Rosc. callus cultures, increasing levels of YE (250, 500 and 750 mg/L) led to favorable activity in the growth of callus on the scope of the fresh and dry weight as follows. (2.7, 3.3 and 4.6 g in fresh weight, sequentially) and (0.141, 0.167 and 0.23 g in dry weight, sequentially) and to a decline in 6-gingerol until it was completely undetectable in callus at the level of (750 mg/L). Decreasing YE level from 500 to 250 mg/L increased 6-gingerol levels from 7 to 10 µg/100 mg callus fresh weight [34].

Effect of Salicylic Acid (SA)

Table 8 shows results of using different concentrations (0, 0.5, 1 and 2 mM) of SA on dry weight and tropane alkaloids production from leaf callus of *Atropa belladonna* L for 21 days. SA inhibits callus growth after 8 days and start increase after 15 days but lower than control. Accumulation of both alkaloids atropine and scopolamine in treated callus were inhibited after 8 days and start increase after 15 days but lower than control as illustrated in Figure 6.

In general, SA elicitation has a negative effect on growth. In an earlier study, it was reported that SA treatment also slightly inhibited the growth of *Salvia miltiorrhiza*Bunge. cell cultures[36]. Also, it was found that increasing the SA concentrations in the media strongly suppresse the growth of *Rubia cordifolia* L callus cultures[37].

Effect of Ornithine

Table 9 shows results of using different concentrations (0, 1, 2and 3 mM) of ornithine on dry weight and tropane alkaloids production from leaf callus of *Atropa belladonna* L for 21 days. The dry weight was enhanced after 8 days with all concentration of ornithine. The highest dry weight was recorded with a callus on MS medium supplemented with ornithine at 2 mM. Accumulation of atropine in treated callus was enhanced after 8 days with all concentration of ornithine while scopolamine was decreased. After 15 days, accumulation of atropine and scopolamine in treated callus were increased but lower than control except ornithine at 3mM; atropine concentrations were higher than control. After 21days, accumulation of both alkaloids atropine and scopolamine in treated callus were decreased except ornithine at 1mM, atropine and scopolamine concentrations was higher than control. as illustrated in Figure 7. Boitel-Conti et al. (2000), reported that addition of ornithine as precursors alone (0.5 mmol/L) was ineffective in stimulating hyoscyamine production in transformed root culture of *Datura innoxia* Mill [38].

Table 1: A "0-4 Scale" scoring system developed for measuring the amount of callus obtained[16]

Score	Description
0 (-)	No visible callus
1 (+)	Small proliferation at cut ends only
2(++)	5 mm callus at cut ends
3 (+++)	5-10 mm callus from all over the explant
4(++++)	> 10 mm callus from all over the explant

Table 2: Peak area of atropine sulfate and scopolamine hydrobromide at different concentration at λ 215 nm

Atropine sulfate concentration (mg/L)	Peak area	Scopolamine hydrobromide concentration (mg/L)	Peak area
5	66.5	5	26.4
10	124.7	10	63.5
20	150.9	20	107.9
30	242.2	30	166.5
150	980	150	844.8
250	1760	250	1414.4

Table 3: Effect of different concentration of growth regulators (BA and Kin) with solid medium on branch number and length (cm) of shoot and root from node explants of *Atropa belladonna* L after 4 weeks cultured in MS medium.

Growth regulators	Conc mg/L	Branch number /explant	Length (cm)	
			Shoot	Root
Kinetin (kin)	0	9	15.5	11.75
	0.25	9	15.5	10.5
	0.5	10.25	15	12
	1	8.25	9	4.25
	2	9.5	7.75	4.25
	L.S.D 5%	**2.582**	**5.296**	**2.987**
Benzyl adenine	0	9	15.5	11.75
	0.25	14	9.25	4
	0.5	10.75	6.5	1.5
	1	14	5.75	0.5
	2	16.5	5.25	0.25
	L.S.D 5%	**2.813**	**4.277**	**2.192**

Table 4: Effect of MS medium supplemented with different concentration of 2,4-D and BA in combination on callus formation from different explants (leaf, stem and root) after 21days from cultured.

Growth regulator concentration (mg/L)		Callus formation score		
		Explant types		
BA	**2,4-D**	**Leaf**	**Stem**	**Root**
0.0	**0.0**	-	-	-
0.5		-	-	-
1.0		-	-	-
2.0		-	-	-
0.0	**0.5**	++	++	++
0.5		-	++	++
1.0		+	+	+
2.0		+	++	+
0.0	**1.0**	+++	+++	+++
0.5		-	+	+
1.0		++	+	+
2.0		+	+	-
0.0	**2.0**	+++	+++	+++
0.5		+	++	+
1.0		-	-	-
2.0		-	-	-

Table 5: Effect of 2,4 D concentration on dry weight and production of tropane alkaloids in callus culture from different explants of *Atropa belladonna* L.

2,4-D concentration mg/L	DW (g)				Atropine µg/g dry weight				Scopolamine µg/g dry weight			
	Leaf	Stem	Root	**Mean B**	Leaf	Stem	Root	**Mean B**	Leaf	Stem	Root	**Mean B**
0.25	0.853	1.137	0.983	**0.991**	150.85	176.47	337.67	**221.7**	34.90	68.37	34.93	**46.07**
0.5	1.013	0.963	0.497	**0.824**	376.62	192.70	336.07	**301.8**	103.16	36.61	24.06	**54.61**
1.0	1.057	0.863	1.013	**0.978**	190.00	73.79	135.27	**133.0**	44.51	9.77	13.69	**22.66**
2.0	0.717	0.820	0.273	**0.603**	55.67	162.64	1.29	**73.20**	10.24	27.87	1.91	**13.34**
Mean A	**0.910**	**0.946**	**0.692**		**193.3**	**151.4**	**202.6**		**48.20**	**35.66**	**18.65**	
L.S.D 5%												
(A)	**0.02677**				**8.381**				**3.137**			
(B)	**0.03092**				**9.677**				**3.622**			
(AB)	**0.05355**				**16.76**				**6.274**			

Table 6: Effect of jasmonic acid concentration on dry weight and production of tropane alkaloids in callus culture of *Atropa belladonna* L.

JA Concentration (µM)	DW (g)				Atropine µg/g dry weight				Scopolamine µg/g dry weight			
	Time in days			Mean B	Time in days			Mean B	Time in days			Mean B
	8	15	21		8	15	21		8	15	21	
0	0.165	0.203	0.210	**0.1928**	27.1	131.0	151.5	**103.2**	18.6	45.2	39.7	**34.49**
10	0.200	0.225	0.192	**0.2057**	36.0	81.5	65.2	**60.90**	1.7	21.9	17.8	**13.82**
25	0.166	0.211	0.193	**0.1897**	91.5	130.2	42.3	**87.98**	2.1	33.8	22.9	**19.60**
50	0.155	0.199	0.212	**0.1883**	157.0	19.5	97.8	**91.44**	31.7	1.8	27.7	**20.40**
Mean A	**0.1714**	**0.2092**	**0.2017**		**77.90**	**90.57**	**89.18**		**13.53**	**25.65**	**27.05**	
L.S.D 5% (A)	**0.008467**				**3.341**				**1.305**			
(B)	**0.009776**				**3.858**				**1.507**			
(AB)	**0.01693**				**6.683**				**2.610**			

Table 7: Effect of yeast extract concentration on dry weight and production of tropane alkaloids in callus culture of *Atropa belladonna* L.

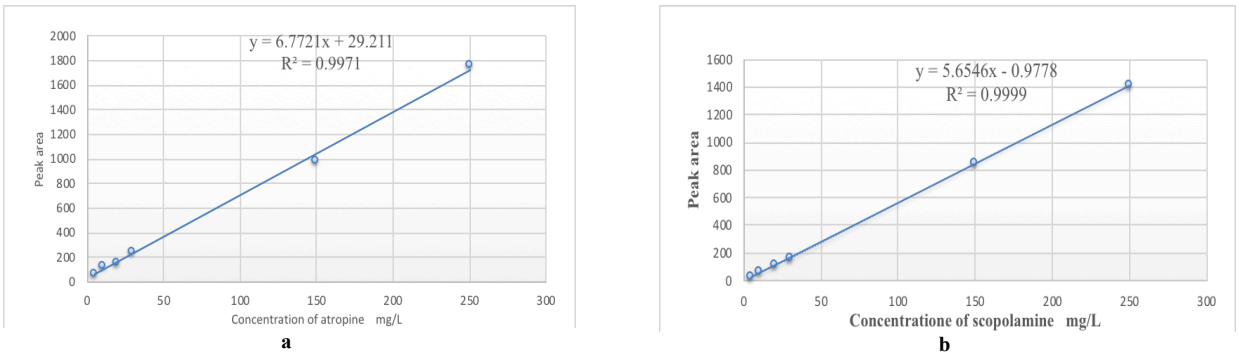
YE Concentration (g/L)	DW (g)				Atropine µg/g dry weight				Scopolamine µg/g dry weight			
	Time in days			Mean B	Time in days			Mean B	Time in days			Mean B
	8	15	21		8	15	21		8	15	21	
0	0.165	0.203	0.210	**0.193**	27.1	131.0	151.5	**103.2**	18.6	45.2	39.7	**34.49**
0.5	0.206	0.188	0.271	**0.222**	31.5	178.2	84.9	**98.22**	1.3	46.5	19.7	**22.48**
1	0.185	0.379	0.231	**0.265**	29.4	173.2	101.1	**101.2**	1.4	41.4	23.7	**22.18**
2	0.176	0.148	0.231	**0.185**	5.3	14.6	36.7	**18.87**	1.5	1.8	27.4	**10.22**
Mean A	**0.183**	**0.229**	**0.236**		**23.32**	**124.3**	**93.56**		**5.700**	**33.68**	**27.64**	
L.S.D 5% (A)	**0.008467**				**3.795**				**1.538**			
(B)	**0.009776**				**4.382**				**1.776**			
(AB)	**0.01693**				**7.590**				**3.077**			

Table 8: Effect of salicylic acid concentration on dry weight and production of tropane alkaloids in callus culture of *Atropa belladonna* L.

SA Concentration (mM)	DW (g)				Atropine µg/g dry weight				Scopolamine µg/g dry weight			
	Time in days			Mean B	Time in days			Mean B	Time in days			Mean B
	8	15	21		8	15	21		8	15	21	
0	0.165	0.203	0.210	**0.193**	27.10	131	151.5	**103.2**	18.63	45.13	39.7	**34.49**
0.5	0.206	0.164	0.165	**0.178**	6.567	0.1	34.5	**13.74**	1.267	1.567	16.3	**6.367**
1	0.147	0.203	0.166	**0.172**	0.1000	8.2	48.2	**18.84**	1.733	13.07	30.3	**15.03**
2	0.125	0.153	0.181	**0.153**	0.1667	3.3	23.7	**9.056**	2.100	1.700	16.2	**6.656**
Mean A	**0.160**	**0.181**	**0.181**		**8.483**	**35.66**	**64.50**		**5.933**	**15.37**	**25.61**	
L.S.D 5% (A)	**0.008467**				**2.859**				**1.259**			
(B)	**0.009776**				**3.301**				**1.454**			
(AB)	**0.01693**				**5.718**				**2.518**			

Table 9: Effect of ornithine concentration on dry weight and production of tropane alkaloids in callus culture of *Atropa belladonna* L.

Ornithine Concentration (mM)	DW (g)				Atropine µg/g dry weight				Scopolamine µg/g dry weight			
	Time in days			Mean B	Time in days			Mean B	Time in days			Mean B
	8	15	21		8	15	21		8	15	21	
0	0.165	0.203	0.210	**0.1928**	27.1	131.0	151.5	**103.2**	18.6	45.2	39.7	**34.49**
1	0.212	0.211	0.186	**0.2031**	41.0	117.6	236.9	**131.8**	0.8	25.4	43.1	**23.10**
2	0.244	0.225	0.247	**0.2388**	51.6	129.7	93.9	**91.72**	0.7	35.1	28.2	**21.33**
3	0.160	0.210	0.220	**0.1966**	51.7	140.1	104.7	**98.81**	1.1	42.9	38.6	**27.51**
Mean A	**0.1952**	**0.2123**	**0.2159**		**42.83**	**129.6**	**146.8**		**5.308**	**37.13**	**37.39**	
L.S.D 5% (A)	**0.008467**				**2.835**				**1.394**			
(B)	**0.009776**				**3.274**				**1.610**			
(AB)	**0.01693**				**5.670**				**2.789**			



a **b**

Figure 1: Calibration curve of atropine sulfate (a) and scopolamine hydrobromide (b)

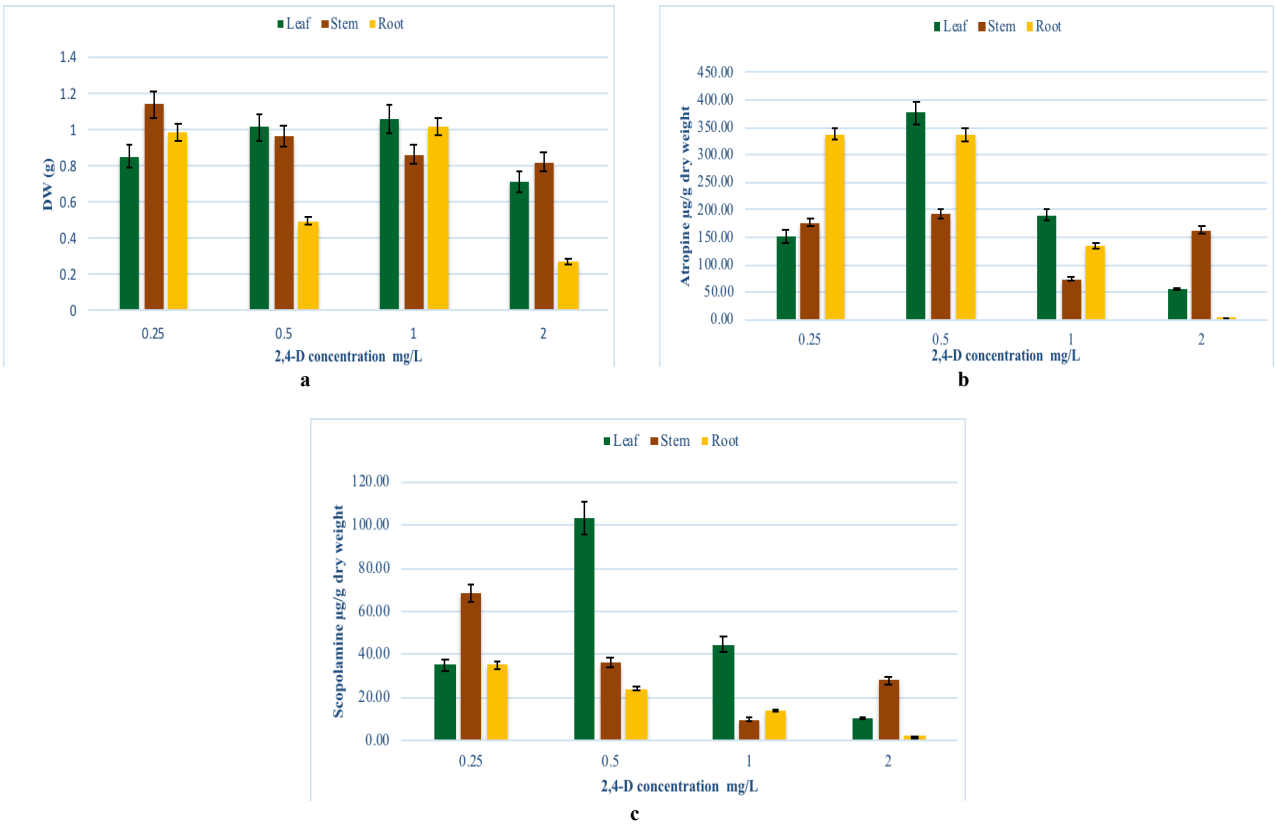


Figure 2: Effect of 2,4 D concentration on dry weight (a) and atropine (b) and scopolamine (c) contents of callus culture from different explants (leaf, stem, and root) of *Atropa belladonna* L.

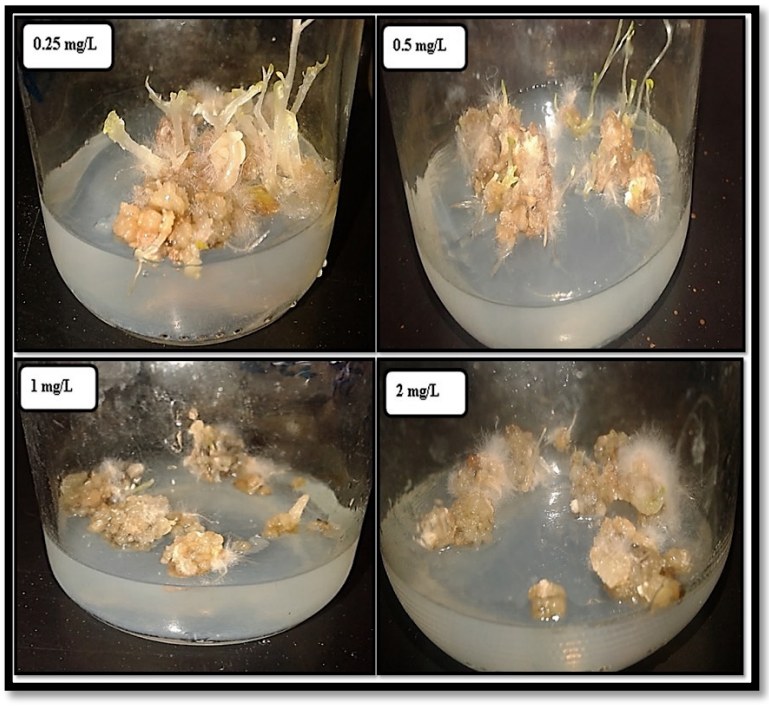


Figure 3: *Atropa belladonna* L leaf callus initiated on different concentration of 2,4 D on solid MS media (1920x2560 pixels)

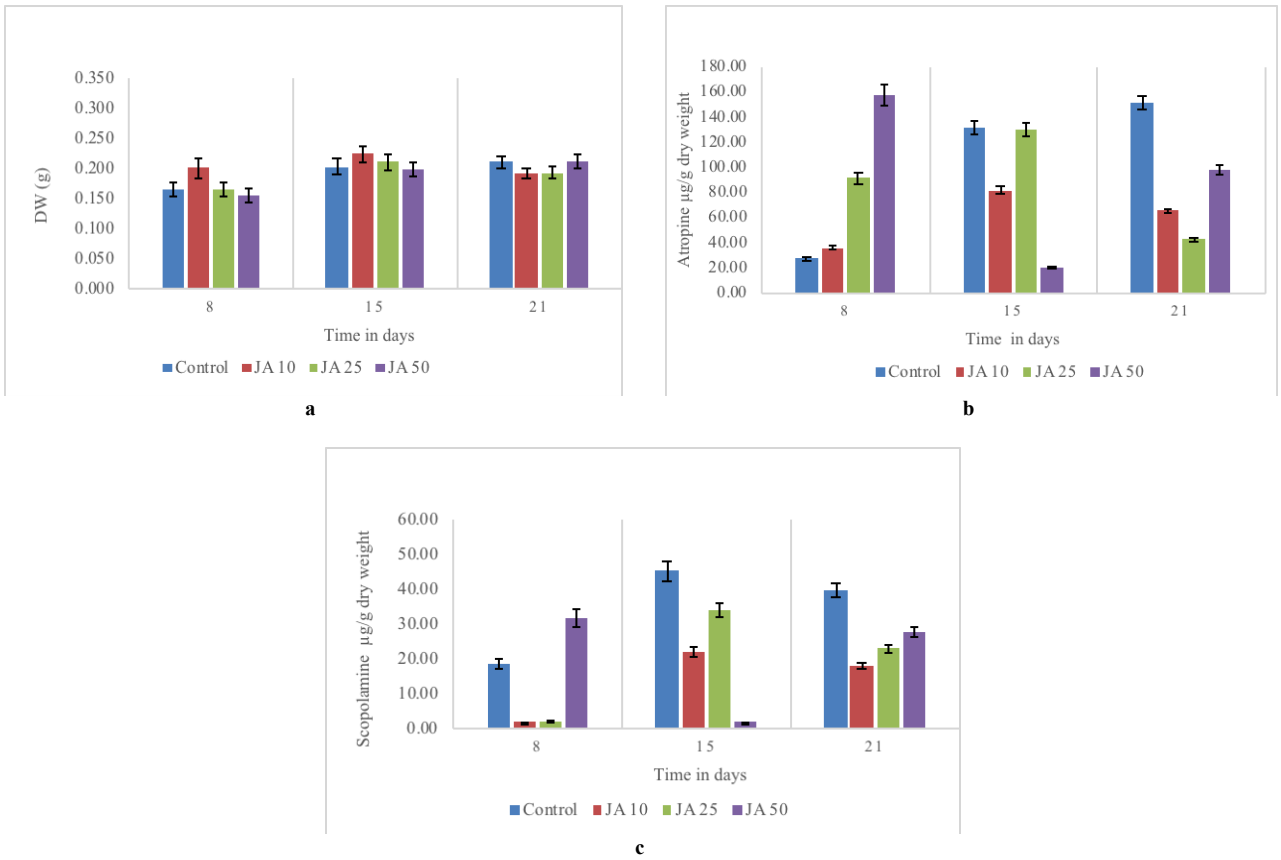


Figure 4: Effect of jasmonic acid concentration on dry weight (a) and atropine (b) and scopolamine (c) contents of callus culture of *Atropa belladonna* L.

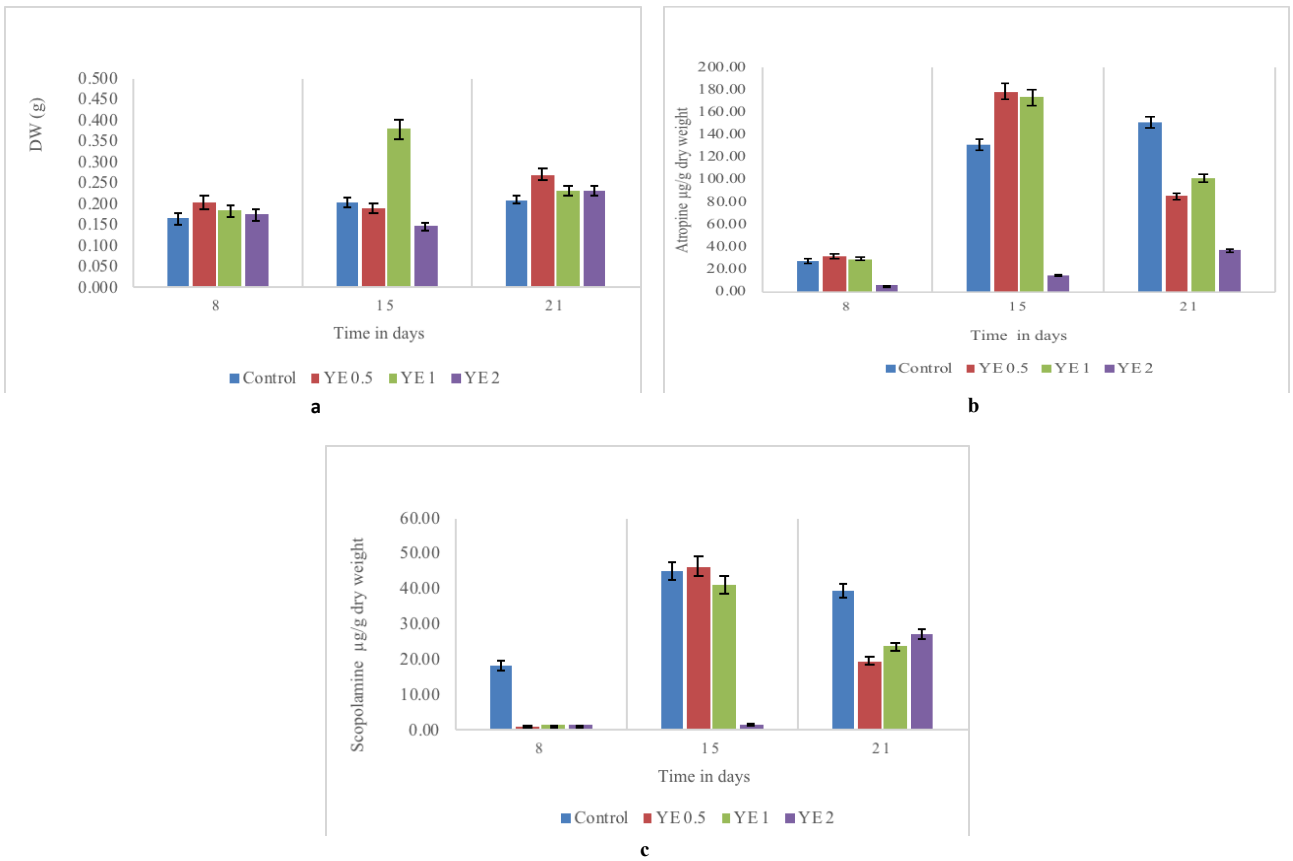


Figure 5: Effect of yeast extract concentration on dry weight (a) and atropine (b) and scopolamine (c) contents of callus culture of *Atropa belladonna* L.

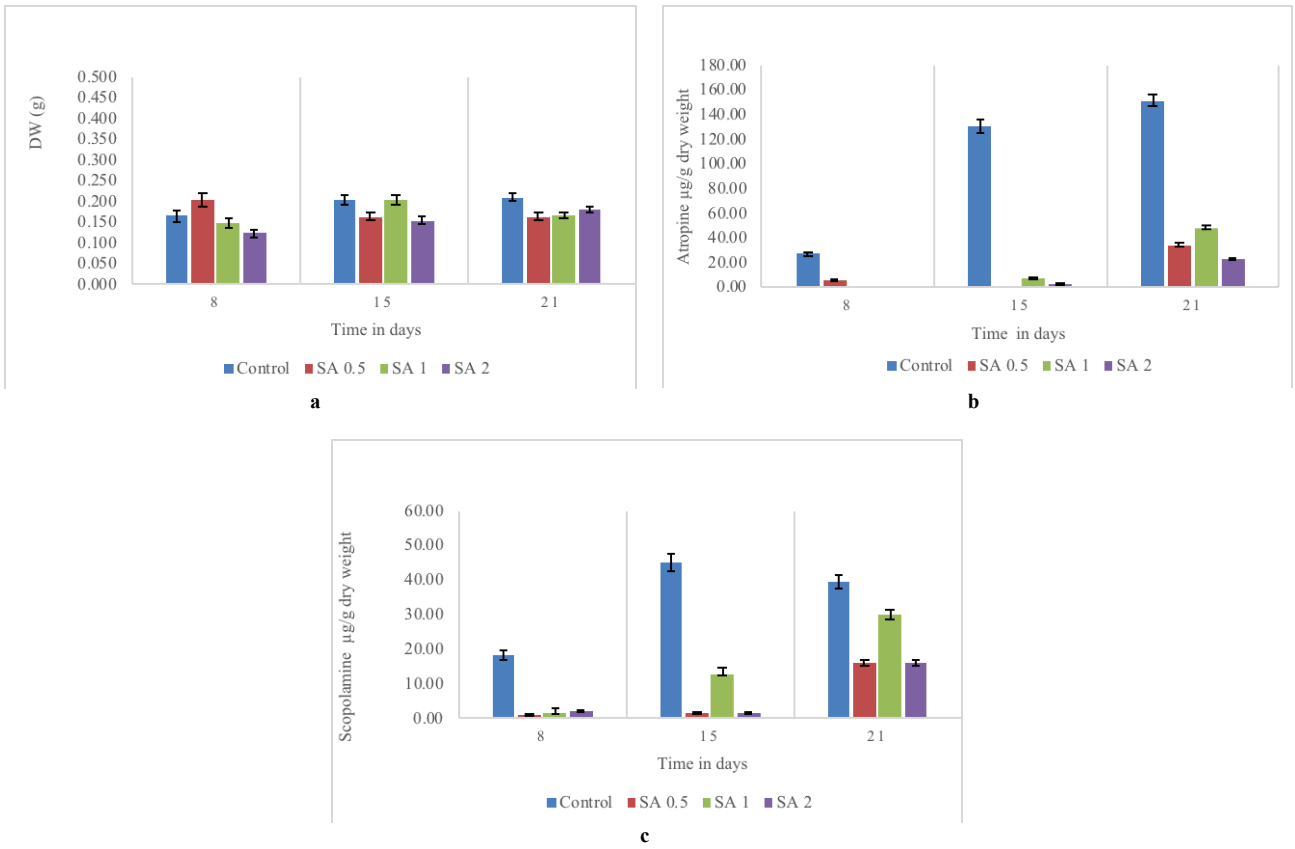


Figure 6: Effect of salicylic acid concentration on dry weight (a) and atropine (b) and scopolamine (c) contents of callus culture of *Atropa belladonna* L.

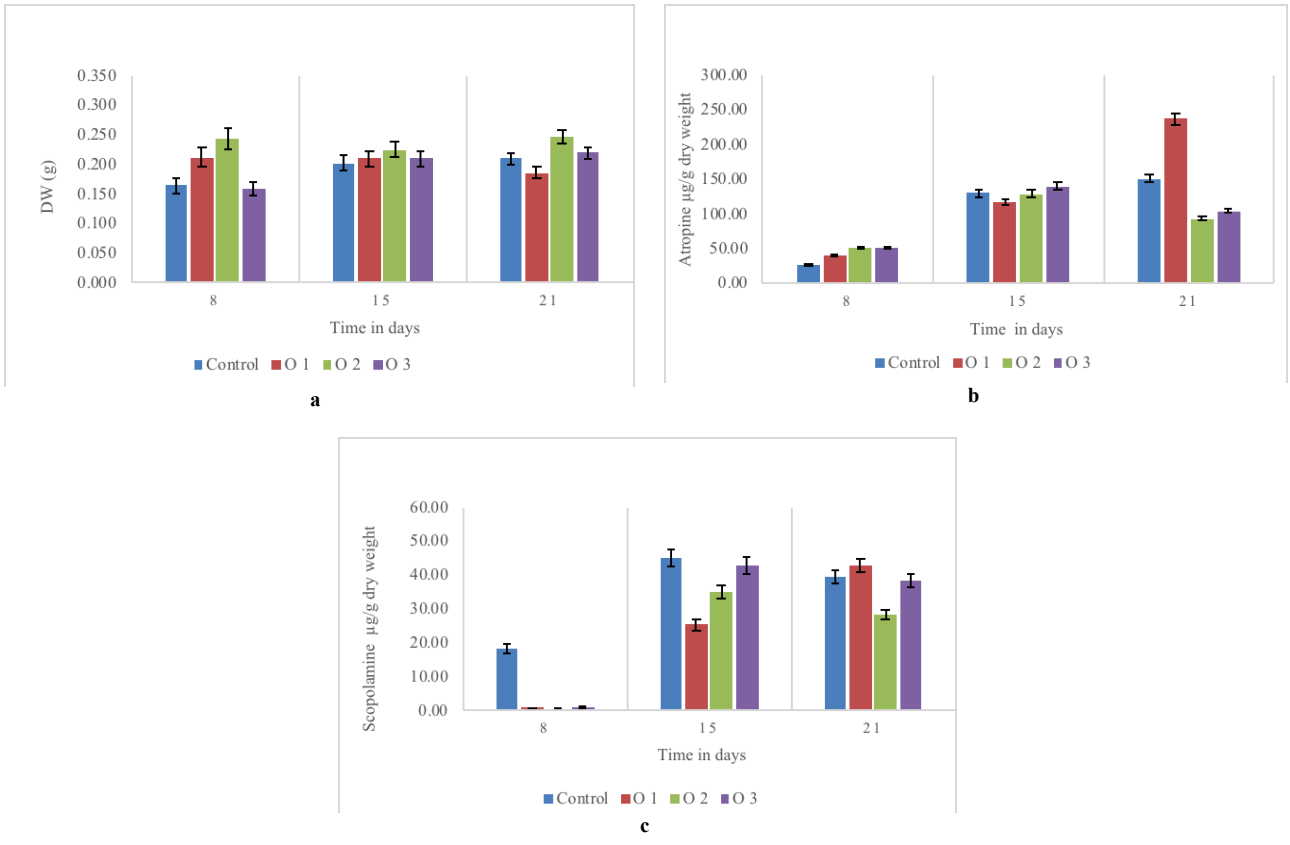


Figure 7: Effect of ornithine concentration on dry weight (a) and atropine (b) and scopolamine (c) contents of callus culture of *Atropa belladonna* L

CONCLUSION

This work shows that 2,4-D alone at 1mg/L and 2 mg/L gave the highest callus formation score. The highest concentration of atropine (376.62 µg/g DW) and scopolamine (103.16 µg/g DW) were obtained from leaf callus on MS medium supplemented with 2,4 D at 0.5 mg/L. Accumulation of both alkaloids atropine and scopolamine in callus shows higher concentration after 21days with ornithine at 1mM in comparison with control callus. Salicylic acid inhibited callus growth and accumulation of atropine and scopolamine in treated callus.

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