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EFFECT OF DIFFERENT CONCENTRATIONS OF PLANT HORMONES ON SEED GERMINATION AND CALLUS INDUCTION OF *ATROPA BELLADONNA* IN *IN VITRO* CONDITIONS

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Abstract

Belladonna (*Atropa belladonna*) is an important medicinal plant of the family Solanaceae. The combination between auxins and cytokinins is used in plant tissue culture to stimulate plant cell elongation, division, and callus formation. The study was conducted at the tissue culture lab, College of Agriculture, University of Baghdad in two experiments to determine the best concentration of HCL can enhance the seed germination, the best combination between auxin and cytokinin, and plant part leading to the best callus induction. Three concentrations of HCL (0, 25, and 50%), three periods of time (0, 5 and 10 minutes) for soaking seeds, and three concentrations of GA3 added to the media were used. The combination of 50% HCl, soaking for 5 minutes, and 0.6 mg/L GA3 gave the highest percentage of germination (100%). Callus induction affected significantly by, auxin, cytokinins, and x-plant part. Three concentrations of 2,4-D (0, 1.5, 3.0, and 4.0 mg. L^{-1}), three concentrations of BA (0.0, 0.2, and 0.8 mg. L^{-1}), and three parts of an ex-plant (shoot tips, cotyledon leaves, and hypocotyl leaves) were used. The combination of 1.5 mg.L^{-1} of 2, 4-D, 0.8 mg.L^{-1} of BA and shoot tip of the ex-plant was the best in giving the highest weight of fresh and dry callus (167.12 and 17.63 mg, respectively).

Key words : Belladonna (*Atropa belladonna*), soaking seeds in HCL, 2, 4-D, BA, GA3, part of ex-plant.

Introduction

Belladonna (*Atropa belladonna*) is one of the important medicinal plants of the family Solanaceae used in medicine and pharmacy. The most important effective alkaline components of the Belladonna are Atropine and Hyoscyamine, which works as a pain reliever, a sedative and inhibitor for the central nervous system. Belladonna plants grow in the form of shrubs, characterized by 1.5 m height, cone roots, the leaves are oval in shape, the flowers are small in length of 3.5 and 2.5 cm, and by their small, spherical, soft, and yellowish fruits (Aldejwi, 1996).

Belladonna seeds are slow in germination due to the hardness and thickness of the seed coats, therefore, the seeds are treated in different ways prior to planting to overcome the hardness of the seeds and help to absorb water for germination (Qutub, 1979; Shofali, 2010). Abdel-Hady *et al*. (2008) referred to the possibility of increasing the germination percent of belladonna seeds by using GA3 at concentrations of (20, 40, 60, 80, 100 and 200 p.p.m), and found an increment in the percentage of germination related to the GA3 concentration increase till the 100 p.p.m.

Auxins are compounds widely used in plant tissue culture to stimulate plant cell elongation and division, and callus formation when they interact with cytokinins. 2,4-D is one of the most effective auxins that induce callus formation and inhibits organ formation (Hopkins, 1999).

Callus is an undifferentiated tissue arises as a result of injuries occur to plant tissues and organs (Mohemad and Salih, 1990; George and Sherrington, 1993). They explained that callus should be incubated in darkness, due to the fact that darkness prevents light - sensitive compounds, such as internal hormones like auxins, from oxidation furthermore it makes the cell walls thinner and less thickness which in turn increases their permeability for materials like growth regulators found inside the cultured tissues then increases the response of plant parts

for callus induction (Hedden and Stephen, 2006). The callus tissue is produced from the outer cortical cells of the plant part, where the continuous division of these cells will lead to callus formation on this part of the plant (Ramawat, 2004).

Different kinds callus may be obtained based on the explant. It may be solid or brittle in textures and green, yellow or white in color (Fahmi, 2003). Al-Mukhtar (2008) reported that the shoot tip was the superior part of the plant to induce callus in comparison to the cotyledon leaf and hypocotyl of poppy (*Papaver somniferum*). Al-Saedi (2011) during studying belladonna, concluded that the shoot tip was the best part of plant for inducing callus.

Materials and Methods

The study was conducted at the tissue culture lab/ College of Agriculture, University of Baghdad. It included two experiments.

First experiment : The disinfestation and germination of belladonna seeds

Belladonna seeds were disinfested by 4.5% of sodium hypochlorite (NaOCl) for 15 minutes, then rinsed with distilled sterilized water three times to get rid of the disinfectant residue (Al-Marsomi, 2010; Al-Saedi, 2011) and each 80–100 seeds were treated by three concentrations of HCL (0, 25 and 50%) for three periods of time (0, 5 and 10 minutes) to enhance the seed germination. After that, the seeds were cultured in MS media (Murashige and Skoog, 1962) supplied with vitamins and GA3 at three concentrations (0.0, 0.2, 0.4, and 0.6 mg. L^{-1}). Finally, the cultures were arranged into ten replicates for each treatment and incubated at 25 ± 2ºC. After seven days, the germination rate was calculated.

Second experiment: Callus induction

To prepare media devoted to callus induction, MS salts and vitamins were dissolved, next combinations of plant regulators 2,4-D (0, 1.5, 3.0, and 4.0 mg. L^{-1}) and BA (0.0, 0.2, and 0.8 mg. L^{-1})were added. Explant parts (shoot tips, cotyledon leaves, and hypocotyl leaves) grown from the disinfested seeds were cultured to determine the best plant regulator concentration and best explant part in inducing callus. Each treatment was replicated ten times.

Fresh and dry weights of callus were recorded seven days after culturing. The callus pieces were picked and the remains of adhered media were removed, the callus pieces were weighed, dried at 70 C by the oven for 24 hours and weighed again to calculate the dry weight.

Complete Randomized Design (CRD) as a factorial experiment of ten replicates was used to compare among the treatments depending upon the Least Significant Difference (LSD) test under the significance level of 0.05.

Results and Discussion

Both HCl and GA3 concentrations were significantly effective in the percentage seed germination. The concentration 50% HCl was significantly superior to the other concentrations in the germination percent (75%), while the concentration 25% HCl gave a less germination percent (46%). GA3 at the concentration of 0.4 mg/L was significantly superior to the other AG3 concentrations in the percentage of seed germination (47%);however, this concentration did not significantly differ from the concentration 0.6 mg/L AG3 that resulted in germinating 46% of seeds (table 1).

The interaction between HCl concentration and GA3 concentration affected significantly the percentage of seed germination. The treatment of 50% HCl with 0.6 mg/L GA3 gave the highest percentage of seed germination (70%) that differed significantly from all other treatments followed by the treatment of 25% HCl with 0.4 mg/L in which 63% of the seeds were germinated. On the other hand, in the control treatment, the percentage of seed germination was negligible (0%).

Table 1 : Effect of HCl, GA3 and their interactions on the percentage of belladonna seed germination.

HCl concentration Mg.L^{-1}	GA_3 concentrationMg.L^{-1}				Mean
	0.0	0.2	0.4	0.6	
0	0	20	20	33	18
25	33	53	63	33	46
50	63	37	57	70	57
LSD	0.230				0.109
Mean	32	37	47	46	
LSD	0.126				

Table 2 : Effect of HCl concentrations, the time period and their interaction on the percentage of seed germination.

HCl concentration (Mg.L^{-1})	Germination percent %		Mean
	5 Min	10 Min	
0	18	18	18
25	63	28	46
50	68	45	57
LSD	0.162		0.089
Mean	50	31	
LSD	0.109		

The germination percentage of belladonna seeds was significantly affected by the period of soaking them in HCl and the interaction between the soaking period and the HCl concentration (table 2). The period of 5 minutes was superior in giving the highest germination percentage (50%) compared to the period of 10 minutes that gave only 31%. The interaction between the soaking for 5 minutes and the 50% HCl affected significantly giving the highest percentage of belladonna seed germination (68%) in comparison to the interaction between the period of 10 minutes with HCl at 50% and between the period 10 minutes and 25% HCl, where the seed germination percentages were 45 and 28%, respectively; furthermore the least percentages of seed germination was 18 % for both periods (5 and 10 minutes) when the seed soaked in water only (0% HCl).

The seed germination was also significantly affected by the GA3 concentrations in the media; as well as, by the interaction between its concentration and the period of soaking the seeds in HCl. The GA3 concentration 0.4 mg/L surpassed the other concentrations by giving the highest germination percentage (47%); whereas, the other two concentrations (0 and 0.2 mg/L) gave only 32 and 37%, respectively. There was a significant effect of the interaction between the period of soaking in the HCL and the GA3 concentration. The percentage of seed germination increased in combination with the increase of GA3 concentration when the seeds were soaked in HCl for 5 minutes, thus the highest percentage of this interaction (58%) was got from the period of 5 minutes with the 0.6 mg/L AG3; whereas, the germination percentage decreased at the high concentration of GA3 (0.6 mg/L) when the period of soaking in HCl was 10 minutes. However, these values were different significantly from the less one gotten from the interaction between 10 minute period and 0.0 mg/L AG3.

The effect of the tribal interaction including HCl concentrations, the soaking seed period and GA3 concentrations was significant in the seed germination percentage (table 4). The interaction of 50% HCl, soaking for 5 minutes, and 0.6 mg/L AG3 was superior in the percentage of seed germination giving 100% followed by the interaction of 25% HCl, soaking for 5 minutes, and 0.4mg/L AG3 giving 93%; whereas, the least percentage of belladonna seed germination was gotten from the interaction of 25% HCl, soaking the seeds for 10 minutes, and 0mg/L AG3 that was 13%.

Several researchers have reported that Gibberellins stimulated dormant and non-dormant seeds regardless the cause of dormancy. It stimulated germination of seeds whose embryo was incomplete or their dormancy caused by the presence of inhibitory substances in the embryo or other different seed parts. In addition, there were many studies indicated that treating seeds by coldness led to increased the levels of substances similar to Gibberellins, and decrease the level of ABA in those seeds, which eventually led to the seed germination at the end of the treatment period (Salman, 1988; Taiz and Zeiger, 2006).

Table 3 : Effect of GA3 concentrations, the time period and their interaction on the percentage of seed germination.

Time	GA3 Concentration				Mean
	0.0	0.2	0.4	0.6	
5 Min	44	47	51	58	18
10 Min	20	27	42	33	46
LSD	0.199				0.089
Mean	32	37	47	46	
LSD	0.126				

Table 4 : Effect of GA3 concentrations, HCl concentrations, the time period, and their interaction on the percentage of seed germination.

HCl concentration ($Mg.L^{-1}$)	Time	GA_3 concentration ($Mg.L^{-1}$)				Mean
		0.0	0.2	0.4	0.6	
0	5	0	20	20	33	18
	10	0	20	20	33	
25	5	53	67	93	40	46
	10	13	40	33	27	
50	5	80	53	40	100	57
	10	47	20	73	40	
LSD		0.308				0.089
Mean		32	37	47	46	
LSD		0.126				

These results were consistent with the findings of Ruminska *et al*. (1978), which indicated that soaking seeds in solutions containing concentrations of GA3 (2000, 1500, 1000, 500) ppm increased the ability of different species seeds, including belladonna, to germination; as well as it induces proliferation.

Callus induction

The induced callus was significantly affected by the hormone type and concentration; so, the auxin, 2, 4- D, and Cytokinin, BA, affected the fresh and dry weight of the callus differently (table 5). The concentration 1.5 mg/L 2, 4-D was superior to the other concentrations giving the highest averages of fresh and dry weight (64.35 and 6.96 mg, respectively). Increasing the 2, 4- D led to a decline in the callus weight; consequently, the least fresh

and dry weight (14.29 and 1.51 mg, respectively) came from the highest concentration of the auxin, 2, 4- D (4.5 mg/L). On the other hand, the cytokinin, BA, affected the callus weight in a different way; increasing the concentration was combined by increasing the callus weight, so, the highest fresh and dry weights (40.42 and 4.42 mg, respectively) were obtained from the highest concentration of BA (0.8 mg/L), as a result it differed significantly from the less concentration (0.2 mg/L)that gave 37.23 and 3.96 mg, respectively.

The effect of interaction between the 2, 4-D and BA was also significant on the fresh and dry weight of the callus. The results showed that the media supplied with 1.5 mg/L 2, 4-D with 0.8 mg/L BA gave the highest weight of fresh and dry callus (107.92 and 11.96 mg, respectively); thus, this treatment differed significantly from the others followed by the interaction between the treatment of 1.5 mg/L 2, 4–D with 0.2 mg/L BA, the treatment that gave 68.96 and 7.08 mg of fresh and dry weights, respectively.

Inducing callus production was affected significantly by the part of a plant and by the type and concentration of the plant regulators in the media. The plant part and BA concentration significantly influenced the fresh and dry weight of induced callus (table 6). Shoot tip was the best plant part in producing the highest fresh and dry weight (44.23 and 4.6 mg, respectively); and differed significantly from the other two parts whereas, the hypoctyle gave the lowest fresh and dry weight average (15.02 and 1.69 mg, respectively). On the other hand, 0.8 mg/L BA was significantly superior in producing the highest fresh and dry callus weight (40.42 and 4.42 mg respectively) followed by the concentration 0.2 giving 37.23 and 3.96 mg, respectively.

The table also illustrates the superiority of the shoot tip culturing in a media containing 0.8mg.L^{-1} BA giving the highest fresh and dry weights (62.97 and 6.63 mg, respectively). One of the reasons leading to the shoot tip superiority to the other plant parts in this trait is the high concentration of inner auxins in the shoot tip in comparison to the other parts; moreover, the shoot tip, from the view of anatomy consists of active meristematic cells (Saad-Eldin *et al*., 2005; Taiz and Zegier, 2006). These results have been confirmed by Al-Mukhtar (2008), who found that the shoot tips were superior to the other parts of poppy plant (*Papaver somniferum*) giving the highest weight rate of callus; however, they have not agreed with those of Ibrahem *et al*. (2009), who reported that callus induced from cotyledon leaves and hypocotyl of the *Hyoscyamus muticus* L. plants had more weight than

Table 5 : Effect of 2,4-d and BA concentrations and their interactions on the callus induced from different parts of belladonna plant on the **fresh weight**.

2,4-D concentration (mg.L^{-1})	BA concentration (mg.L^{-1})			Mean
	0.0	0.2	0.8	
0	0.00	0.00	0.00	0.00
1.5	16.17	68.96	107.92	64.35
3.0	18.17	56.50	37.25	37.31
4.5	2.92	23.46	16.50	14.29
LSD	6.112			3.529
Mean	9.31	37.23	40.42	
LSD	0.342			

A-on the dry weight

2,4-D concentration (mg.L^{-1})	BA concentration (mg.L^{-1})			Mean
	0.0	0.2	0.8	
0	0.00	0.00	0.00	0.00
1.5	1.83	7.08	11.96	6.96
3.0	1.88	6.21	4.04	4.04
4.5	0.33	2.54	1.67	1.51
LSD	0.684			0.395
Mean	1.01	3.96	4.42	
LSD	0.342			

that got from the shoot tip.

The effect of 2,4-D, in addition to the type of ex-plant part, as well as their interactions were significant on the fresh and dry weight of the induced callus after five weeks of culture (table 7). The concentration 1.5 mg. L^{-1} 2,4-D was significantly superior to the other concentrations in the fresh and dry weight of callus (64.35 and 6.96 mg, respectively) followed by the concentration 3.0 mg. L^{-1} 2, 4-D producing 37.31 and 4.04 g, respectively. The shoot tip differed significantly from the other part of the plant; it was the best in giving the highest fresh and dry weight of callus (44.23 and 4.61 mg respectively) followed by the cotyledon leaves (27.71 and 3.08 mg, respectively).

The interaction between the 2, 4-D concentration and the part type the ex-plant was also significant in its effect on the fresh and dry weight of the induced callus. The results of the same table showed the significant superiority of the shoot tip cultured in a media supplied with 1.5 mg. L^{-1} 2, 4-D giving the highest values of fresh and dry callus weight (104.67 and 10.92 mg respectively) followed by the cotyledon leaves cultured in media contained 1.5 mg. L^{-1} 2, 4-D producing 62.25 and 7.04 as fresh and dry weight respectively; on the other hand, culturing the cotyledon leaves in media contained 4.5mg.

Table 6 : Effect of BA concentration, the plant part and their interactions on the callus weight.

A-Fresh weight

BA concentration ($mg.L^{-1}$)	Plant Part			Mean
	Shoot tip	Cotyledon leaves	Hypoctyl	
0	15.94	10.03	1.97	9.31
0.2	53.78	37.69	20.22	37.23
0.8	62.97	35.41	22.87	40.42
LSD	5.293			3.056
Mean	44.23	27.71	15.02	
LSD	3.056			

B- Dry weight

BA concentration ($mg.L^{-1}$)	Plant Part			Mean
	Shoot tip	Cotyledon leaves	Hypoctyl	
0	1.66	1.13	0.25	1.01
0.2	5.56	4.00	2.31	3.96
0.8	6.63	4.13	2.50	4.42
LSD	0.593			0.342
Mean	4.61	3.08	1.69	
LSD	0.342			

L^{-1} 2, 4-D resulted in the lowest values of fresh and dry weight (9.21 and 104 mg, respectively).

The tribal interaction included BA concentrations, 2,4-D concentrations and the type of ex-plant part affected significantly the fresh and dry weight of the induced callus (table 8). Using the BA in different concentrations with different type of plant parts, without adding 2,4-D to the media, did not show any response, while culturing the shoot tip in media supplied with 0.8 mg. L^{-1} AB, in addition to 1.5 mg.L^{-1} 2,4-D, induced the highest fresh and dry weight of callus (167.12 and 17.63 mg respectively), followed by the interaction consisted of the shoot tip, 0.2 mg. L^{-1} AB and 1.5 mg. L^{-1} 2,4-D (118.12 and 11.88 mg, respectively). The culture media used for inducing callus from the cotyledon leaves produced 97.00 mg fresh weight and 11.75 mg dry weight when they were supplied with 0.8 mg. L^{-1} AB and 1.5 mg. L^{-1} 2,4-D; likewise, the same media composition produced the highest fresh and dry weight of callus (59.63 and 6.50 mg respectively) when the hypocotyls were used.

The lowest fresh and dry weight of induced callus were resulted from: culturing the cotyledon leaves in media contained 0.0 mg. L^{-1} AB and 4.5 mg. L^{-1} 2, 4-D (2.50 and 0.25 mg, respectively), culturing the shoot tip in the same media (6.25 and 0.75 mg respectively), and culturing the hypocotyl in media contained 0.0 mg. L^{-1} AB and 3.0 mg. L^{-1} 2, 4-D(7.88 and 1.00 mg respectively); however, these three weights were not different significantly from each other.

Table 7 : Effect of 2,4-D concentration, the plant part and their interactions on the callus weight.

A-Fresh weight

2,4-D concentration ($mg.L^{-1}$)	Plant Part			Mean
	Shoot tip	Cotyledon leaves	Hypoctyl	
0	0.00	0.00	0.00	0.00
1.5	104.67	62.25	26.12	64.35
3.0	51.83	39.38	20.71	37.31
4.5	20.42	9.21	13.25	14.29
LSD	6.112			3.529
Mean	44.23	27.71	15.02	
LSD	3.056			

B- Dry weight

2,4-D concentration ($mg.L^{-1}$)	Plant Part			Mean
	Shoot tip	Cotyledon leaves	Hypoctyl	
0	0.00	0.00	0.00	0.00
1.5	10.92	7.04	2.92	6.96
3.0	5.50	4.25	2.38	4.04
4.5	2.04	1.04	1.46	1.51
LSD	0.684			0.395
Mean	4.61	3.08	1.69	
LSD	0.342			

Results showed that the lower concentrations of 2,4-D were the most effective in inducing callus that may be due to the fact that high concentrations led to reducing the cell division rate (Trigiano and Gray, 2000); furthermore, many researchers reported that high concentration of 2,4-D resulted in the callus tissue death due to the release of ethylene in large quantities leading to rapid divisions of cells and the occurrence of imbalance of growth and then the death of callus tissue (Corchete *et al.*, 1999). It was also observed that high concentrations of auxins inhibited the growth of cells and, conversely, low concentrations promoted the growth of plant cells, as well as affected the middle lamella of cells, which helps to expand the cell wall until the optimal concentration of auxins (Cellaropra and Honkariv, 1984); consequently, 2,4-D is one of the most effective oxygen in preparing cells to divide and form callus (Fahmi, 2003).

It has been observed that the best callus growth is achieved by balancing the concentrations of auxins and cytokinins and the increase in concentrations of either of them at the expense of the other will adversely affect

Table 8 : Effect of 2,4-D concentrations, BA concentrations the plant part and their interactions on the callus weight.

A-Fresh weight

BA concentration ($mg.L^{-1}$)	2,4-D concentration ($mg.L^{-1}$)	Plant Part			Mean
		Shoot tip	Cotyledon leaves	Hypocotyl	
0	**0**	0.00	0.00	0.00	
	1.5	28.75	19.75	0.00	
	3.0	28.75	17.87	7.88	9.31
	4.5	6.25	2.50	0.00	
0.2	**0**	0.00	0.00	0.00	
	1.5	118.12	70.00	18.75	
	3.0	68.25	65.75	35.50	37.23
	4.5	28.75	15.00	26.62	
0.8	**0**	0.00	0.00	0.00	
	1.5	167.12	97.00	59.63	
	3.0	58.50	34.50	18.75	40.42
	4.5	26.25	10.12	13.12	
LSD		10.586			3.056
Mean		44.23	27.71	13.12	
LSD		3.056			

B- Dry weight

BA concentration ($mg.L^{-1}$)	2,4-D concentration ($mg.L^{-1}$)	Plant Part			Mean
		Shoot tip	Cotyledon leaves	Hypocotyl	
0	**0**	0.00	0.00	0.00	
	1.5	3.25	2.25	0.00	
	3.0	2.63	2.00	1.00	1.01
	4.5	0.75	0.25	0.00	
0.2	**0**	0.00	0.00	0.00	
	1.5	11.88	7.13	2.25	
	3.0	7.50	7.13	4.00	3.96
	4.5	2.88	1.75	3.00	
0.8	**0**	0.00	0.00	0.00	
	1.5	17.63	11.75	6.50	
	3.0	6.38	3.63	2.13	4.42
	4.5	2.50	1.13	1.38	
LSD		1.185	0.342		
Mean		1.69	3.08	4.61	
LSD		0.342			

the growth of callus (Hedden and Stephen, 2006; Mino, 1990). BA, in its turn, is the most cytokinins used widely in plant tissue culture due to its strong effect in comparison with Kin and 2ip as a result of presence more than one double band in its structure (Krishnamurty *et al*.,1984). Cytokinin, in the presence of auxins, acts as a key to the initiate the cellular division and the existence of both regulators in the culture media is very important for callus induction (Goodwin, 1985).

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