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RESEARCH ARTICLE

Effect of progesterone as a precursor of the cardiac glycosides and explant type on callus formation and its glycosidal content in *Nerium oleander*

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Abstract

In vitro experiment has been conducted to evaluate the effect of different concentrations of progesterone and explants type on glycosidal content in *Nerium oleander*. Obtained callus of leaves and shoot tips was cultured on ¾ MS medium supported by 2 mg/l. kinetin combined with 2 mg/l. 2,4-D, different levels of (0, 0.2, 0.5, 1 and 1.5 mg/l.) progesterone, 6 g./l. agar, and 30 g/l. sucrose for 6 weeks. Our results revealed that the highest fresh and dry weights of callus were achieved from the medium contained 0.5 mg/l. progesterone as for shoot-tips and leaves calli which averaged 3.145 and 0.300 g. However, this concentration gave low percentage of oleandrin (0.7%) comparing with the percentage of 0.8% which achieved from the medium supported with the high concentration of progesterone (1 mg/l.), it gave the highest value of oleandrin quantity either in callus derived from shoot-tip or leaf (1.96 and 2.24 mg.).

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INTRODUCTION

The techniques of tissue cultures attracted the attention of several investigators since the beginning of this century, and become of important value only during recent decades. They are now established as potent research tool having a lot of values: The most important one of them is tissue cultures can be used for feeding experiments in conjunction with label compounds, and are also useful for the determination of the site of synthesis of particular compounds. Accordingly, the authors thought that the applications of this branch of science on medicinal plants is of great value, particularly if it is carried out on the plants found in Egypt like oleander, *Nerium oleander* L. family Apocynaceae (Hegi, 1927).

Various parts of the plants are reputed to be therapeutic agents in the treatment of swellings, leprosy, and eye and skin diseases. The leaves mainly possess cardio tonic and antibacterial properties and are utilized as counter-poisons against snake venom (Dymock et al., 1891; Chopra et al., 1956). Because of these applications and their pharmacological effects, many researchers have investigated the various compounds of *Nerium* spp. The substances which have been found in the leaves, root bark and seeds belong to the flavonoids, saponins, carelenolides, pregnanes and hexadecanoic acid derivatives. The leaves are used for the preparation of the steroidal glycoside oleandrin which is considered as the principal drug available for treatment of carelio-vascular disorder (Tittel and Wagner, 1981; and Abe and Yamauchi, 1992).

Progesterone, which is formed, with cardiac glycosides, as a result of feeding pregnanolone, is itself a precursor of the cardiac glycosides. Evans (1989) mentioned that aglycones of the cardiac glycosides are derived from mevalonic acid and progesterone, which is formed, with cardiac glycosides, in *Digitalis lanata* as a result of feeding pregnenolone, is itself a precursor of the cardiac glycosides. *Catharanthus roseus* plants are the source of pharmaceutically important alkaloids such as vincristine and vinblastine, strictosidine is the precursor

of the majority of monoterpenoid indole alkaloids; it arises from the stereo specific condensation of the indole tryptamine with the iridoid secologanin (Contin et al., 1999). Moreover, the production of podophyllptoxin, a precursor of anti-tumour drugs, was investigated in *Juniperus chinensis* stem derived callus cultures on modified SH medium. The content of podophyllotoxin in the calli grown on the medium was three times higher than that of the intact plant. The production of podophyllotoxin in stem-derived callus cultures were increased by addition of biogenetic precursors, phenylalanine, coniferyl alcohol and a biotic elicitor, chito-oligosaccharides (cos). Podophyllotoxin production was stimulated to 6.4, 3.6, and 2.2 fold by addition of cos, phenylalanine and coniferyl alcohol, respectively (Premjet et al., 2002).

The current research was performed to study the possibility of adding suitable precursor i.e. progesterone in different levels to the culture media aiming to determine its optimum doe which in turn could be used for the commercial production of these valuable metabolites. On the other hand, this study focused on investigating the ability of some external factors such as explant type may alter the callus production and its active ingredient.

MATERIALS AND METHODS

This study was carried out in the tissue culture laboratory of Vegetable and Ornamental Department, Faculty of Agriculture, Mansoura University, Egypt.

Preparation of vegetative explants for in vitro culture:

Small leaves and shoot tips of oleander about (1- 1.5 cm) were excised from shrubs. Before beginning sterilization, any remaining soil or dead parts were removed. This should be followed by washing in tap water containing soap and small drops of Best film. Leaves were sterilized with 30% sodium hypochlorite (10 min.), mercuric chloride 1 g./l. (3 min.) and antioxidants citric and ascorbic acids 0.15 g./l. (15 min.) out of laminar air flow hood. In laminar air flow hood, 70% ethanol (1 min.), mercuric chloride 1 g./L (1 min.), antioxidants (15 min.) and 50% sodium hypochlorite (10 min.). Sterilization of shoot tips out of laminar air flow hood occurred by sodium hypochlorite 30% (10 min.), mercuric chloride 1 g./l. (5 min.) and antioxidants (30 min.). In laminar air flow hood 70% ethanol (1 min.), mercuric chloride 1 g./l. (5 min.), antioxidants (30 min.) and 50% sodium hypochlorite (20 min.).

Cultured media for callus induction:

Three sterilized explants from leaves or shoot tips were cultured in ¾ MS (Murashige and Skoog, 1962) medium. The media were supplemented with 2 mg./l. Kinetin combined with 2 mg./l. 2,4-D, agar was used at 6 g./l. and sucrose at 30 g./l. The media were distributed into 30 clean jars for leaves and shoot tips equally. Each jar contained 30 ml of nutrient media. The media was adjusted to pH 5.7-5.8 before autoclaving for 15 min. at 121^{o}c, 1.5 kg/cm^{3}. All jars were incubated in the growth chamber at 26±2^{o}c and exposed to 16 hr. light/day photoperiod under constant fluorescent light of 1500 Lux. for two weeks.

Experimental treatments:

Callus of leaves and shoot tips was taken out of the jars (cultivated in vitro) and cultured in MS medium supplemented with 2 mg./l. Kinetin combined with 2 mg./l. 2,4-D and different levels of progesterone (0.0, 0.2, 0.5, 1 and 1.5 mg./l.), treatments were five, agar was used at 6 g./l., and sucrose at 30 g./l. The media were distributed into clean jars. Each jar contained 30 ml of nutrient media. The media was adjusted to pH 5.7-5.8 before autoclaving for 15 min. at 121^{o}c, 1.5 kg/cm^{3}. All treatments were incubated in the growth chamber at 26±2^{o}c and exposed to 16 hr. light/day photoperiod under constant fluorescent light of 1500 Lux. Each treatment was represented by 8 jars of four replicates (2 for each), and every jar included 3 pieces of callus. Therefore, treatments were ten as follow:

1. Leaves+0 mg./l. progesterone (control).
2. Leaves+0.2 mg./l. progesterone.
3. Leaves+0.5 mg./l. progesterone.
4. Leaves +1 mg./l. progesterone.
5. Leaves +1.5 mg./l. progesterone.
6. Shoot-tips+0 mg./l. progesterone (control).
7. Shoot-tips +0.2 mg./l. progesterone.
8. Shoot-tips +0.5 mg./l. progesterone.
9. Shoot-tips +1 mg./l. progesterone.
10. Shoot-tips +1.5 mg./l. progesterone.

Experimental design and statistical analysis:

A complete randomize design was used throughout the research. The obtained data were subjected to analysis of variance and the treatment means were compared using L.S.D. test as outlined by Snedecor and Cochran (1975).

Growth measurements:

The data were recorded after 6 weeks from culturing on media for:

1- Fresh weight of callus in g.
2- Dry weight of callus in g.
3- Oleandrin content (percentage and quantity).

Oleandrin determination was performed on callus dry samples derived from leaves and shoot tips explants as well as the same explants from the field (in vivo) for comparison by quantitative TLC-colorimetric method according to Khayal (1970).

RESULTS AND DISCUSSION

Concerning the effect of progesterone levels on fresh weight of callus, it was a matter of interest to notice in Table (1) that fresh weight of callus was significantly increased (3.145 g.) as attributed to the concentration of progesterone (0.5 mg./l.) followed by (1.930 g.) as treated with the concentration of progesterone (1 mg./l.) than the control which was (1.200 g.). The lowest concentration of progesterone (0.2 mg./l.) resulted in lower callus fresh weight (1.480 g.) followed by (1.795 g.) by the concentration of progesterone (1.5 mg./l.), however, the differences were not significant compared with control.

Table (1): Effect of progesterone on *N. oleander* callus fresh weights from shoot tips and leaves.

Type of explant (B) / Progesterone Concentrations (mg./l.) (A)	Average fresh weight of shoot tip callus (g.)	Average fresh weight of leaves callus (g.)	Mean of progesterone conc.
0	1.68	0.72	1.200
0.2	1.52	1.44	1.480
0.5	3.28	3.01	3.145
1	2.25	1.61	1.930
1.5	1.63	1.96	1.795
Mean of explant type	2.072	1.748	
L.S.D. at 5%	(A) 0.750 (B) N.S (A× B) N.S		

Examining the effect of the explant type on fresh weight of callus, data in the same Table showed that although, it was an increment between callus derived from shoot-tip (2.072 g.) and callus derived from leaves (1.748 g.) but, it was non significant. Concerning the interaction between progesterone concentration and the explant type, it was obvious that the highest value of fresh weight (3.28g.) was obtained from callus derived from shoot-tip growth on MS medium supplemented with 0.5 mg./l. progesterone followed by using the same concentration on callus derived from leaves (3.01 g.). The lowest value (0.72 g.) was achieved from callus leaves fresh weight derived from MS medium free of progesterone. These results were on par with those recorded by Khafagi (1999) who mentioned that callus and cell suspension cultures of *Nerium oleander* were initiated from shoot, root and / or hypocotyl explants of aseptically germinated seedlings on Ms medium supplemented with 0.75% agar, 3% sucrose and different concentrations of auxins and cytokinins.

Data in table (2) showed that dry weight of callus was significantly increased up to (0.300 g.) by the concentration of 0.5 mg./l. progesterone than the control which was (0.123 g.) and all other concentrations followed by the level of (1 mg./l.) which gave (0.195 g.) callus dry weight. The level (1.5 mg./l.) progesterone decreased the dry weight of callus than the control to 0.118g. As for the effect of the explant type on dry weight of callus, data in the same table showed that callus derived from shoot-tip was better than callus derived from leaves but, it was non significant differences recorded between average dry weights of callus shoot-tips (0.181g.) and callus leaves (0.171g.).

Concerning the interaction between progesterone concentration and the explant type, it was clear that the highest value of dry weight (0.32g.) was obtained from leaves callus derived from MS medium supplemented with 0.5 mg./l. progesterone followed by treating callus derived from shoot-tip with the same concentration (0.28 g.). The lowest value (0.056 g.) was obtained from shoot-tips callus dry weight derived from MS medium supplemented with the moderate level of progesterone 1.5 mg./l. followed by the value of (0.066 g.) which recorded by leaves callus dry weight derived from MS medium free of progesterone. On the other hand, the lowest concentration (0.2 mg./l.) and the level of (1 mg./l.) had approximately the same effect in increasing the dry weight of leaves callus which averaged (0.14 and 0.15 g.) respectively.

Data presented in table (3) showed that medium supplemented with the high concentration of progesterone (1 mg./l.) and the medium free of the precursor (0 mg./l.) gave the same percentage of oleandrin in callus derived from leaf explant (0.8%). The highest percentage of oleandrin was obtained from callus derived from shoot tip explant grown on medium free of progesterone. The same results were achieved from callus derived from leaf or shoot tip explants grown on medium supplemented with 0.5 mg./l. progesterone. Although, this level gave the highest quantity (2.24 mg.) of oleandrin in callus derived from leaf and (1.96 mg.) in callus derived from shoot tip. Oleandrin was (0.08%/ 1g. dry weight in average) for leaves and shoot tips derived from in vivo plants. It means that oleandrin production was stimulated to 7 fold in callus derived from leaves or shoot tips by addition of 0.5 mg/l. progesterone to MS medium. These results were in accordance with data described by Premjet et al. (2002) who found that the content of podophyllotoxin in the calli grown on the modified SH medium was three times higher than that of the intact plant. On the other hand, our findings were disagreed with results of Rhodes et al. (1994) who illustrated that auxins in the presence of low levels of kinetin induced rapid disorganization of transformed roots of *Nicotiana rustica* ultimately to for suspension cultures of transformed cells and this process was associated with a decrease in nicotine content of cells .

Table (2): Effect of progesterone on *N. oleander* callus dry weights derived from shoot tips and leaves.

Type of explant (B) / **Progesterone Concentrations (mg./l.) (A)**	**Average dry weight of shoot tip callus (g.)**	**Average dry weight of leaves callus (g.)**	**Mean of progesterone conc.**
0	0.18	0.066	0.123
0.2	0.15	0.14	0.145
0.5	0.28	0.32	0.300
1	0.24	0.15	0.195
1.5	0.056	0.18	0.118
Mean of explant type	0.181	0.171	
L.S.D. at 5%	(A) 0.076 (B) N.S (A× B) 0.108		

Table (3) Quantity of secondary product (oleandrin) in dry callus derived from shoot-tips and leaves comparing with in vivo explants.

Treatments	Average dry weight (mg.)		Oleandrin percentage (%)		Oleandrin quantity (mg.)	
	Shoot-tip	Leaf	Shoot-tip	Leaf	Shoot-tip	Leaf
0.0 Progesterone	180	66	0.8	0.8	1.44	0.528
0.5 Progesterone	280	320	0.7	0.7	1.96	2.24
1.0 Progesterone	240	150	0.7	0.8	1.68	1.2
In vivo explants	1000	1000	0.08	0.08	0.8	0.8

These results were in agreement with the findings of Evans (1989) who mentioned that aglycones of cardiac glycosides are derived from mevalonic acid and progesterone which is formed with cardiac glycosides in *Digitalis lanata* as a result of feeding pregnenolone, is itself a precursor of the cardiac glycosides. Similarly, as mentioned by Pedersen et al. (1987**)** who concluded that relatively high levels of caffeine and caffeine precursor in *Caffea arabica* tissue culture appeared at the end of the growth phase in batch culture systems. In another system using *Atropa belladonna* for the production of tropane alkaloids and atropine.

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