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EFFECT OF GROWTH REGULATORS AND ECOTYPES OF EGYPTIAN VINCA ROSA (*Catharanthus roseus*) ON CALLUS INDUCTION AND ALKALOIDS PRODUCTION

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ABSTRACT

Objective of the study is to estimate the effect of growth regulators and different ecotypes of Egyptian *Catharanthus roseus* such as (Ismailia, Fayoum, Alexandria, Sharkia and Cairo) on callus induction and alkaloids production. Different growth regulators combinations were applied. Murashige and Skoog (MS) medium supplemented with four different growth regulators, *i.e*. M1 (2, 4-Dichlorophenoxy acetic acid (2, 4-D) 1.0 µM and Benzyl adenine (BA) 0.5 µM), M2 (2, 4-D 0.5 µM and BA 1.0 µM), M3 (BA 1.0 µM and 1-Naphthaleneacetic acid (NAA) 0.5 µM) and M4 (BA 0.5 µM and NAA 1.0 µM). M1 medium gave the highest value for callus induction frequencies (75%) and callus fresh weight (1.87 g) for produced callus from leaf while the stem produced callus (0.7 g) was the higher too than other mediums. The total alkaloids content (TAC) was estimated using spectrophotometer with Bromocresol green (BCG). The results showed that TAC outperformed in produced callus from stem explant than leaf produced callus, leaf and stem of original plant. The important result showed that the Fayoum ecotype was the highest alkaloid content (2.22 mg/g) for produced callus from stem explant than all studied ecotypes.

Key words: *Catharanthus roseus* ecotypes, total alkaloids content, callus induction.

INTRODUCTION

Medicinal plant is the most exclusive source of life saving drugs for majority of the world's population. They continue to be an important therapeutic aid for alleviating the ailments of human kinds. The search for defence mechanism, longevity and remedies to relieve pain and discomfort drove early man to explore these immediate natural surroundings. It led to the use of plants, animal products and minerals *etc*., and the development of a variety of therapeutic agents (Taha *et al.,* 2009). Today, there is a renewal interest in traditional medicine and an increasing demand for more drugs from plant sources because green medicine is safe and more dependable then costly synthetic drug, many of which have adverse side effects (Sain and Sharma, 2013). Medicinal plants are very important and constitute a valuable natural resource for the nation. According to world researches, more than 3000 plant species are used for pharmaceutical purposes because of their effective compounds. About 21,000 plant species have valuable secondary metabolites meaning that they are classified as medicinal plants (Papadopoulos *et al.,* 2000). By producing alkaloids, some medicinal plants play an important role in curing diseases and are thus a good resource for products associated with health. These effective compounds (secondary metabolites) are synthesized in small amounts by medicinal plants but have great commercial value and command high prices (Papadopoulos *et al.,* 2000; Taha *et al.,* 2009).

Catharanthus is a perennial tropical medicinal plant belongs to the family apocynaceae which comprises eight species, seven endemic to Madagascar (C. *coriaceus*, C. *lanceus*, C. *longifolius*, C. *ovalis*, C. *roseus*, C. *scitulus*, C. *trichophyllus*), and one, C. *pusillus*, from India. Specifically, "Periwinkle" or *Catharanthus roseus* (L.) G. Don (family apocyanaceae), commonly known as "Nayantara" or "Sadabahar",

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is an erect bushy perennial herb and evergreen shrub. The species was formerly known as *Vinca rosea*. The native of "Periwinkle" is mainly Madagaskar. This plant is grown commercially for its medicinal uses in Australia, Africa, India and Southern Europe.There are commonly two varieties of this plant based on the flower colour *viz*., pink flowered (vinca rosa) and white flowered (vinca alba) (Jaleel and Panneerselvam, 2007). The leaves and stem are the sources of the natural dimeric Alkaloids vinblastine (VLB) and vincristine (VCR) that are essential parts of most anti- cancer chemotherapies (Jacobs *et al.*, 2004). *Catharanthus roseus* (L.) G. Don, is an important source of indole alkaloids, which are present in all plant organs. In fact, vinblastine and vincristine are commercial terpenoid indole alkaloids (TIAs) used in anticancer chemotherapy, and together with a number of related semi-synthetic compounds, are collectively named the vinca alkaloids. Currently, vinblastine, vincristine, vinorelbine and vindesine have been used in clinical trials, although only vinblastine, vincristine and vinorelbine have been approved for medical treatment in the United States (Rowinsky, 2003).Vincristine and vinblastine also show a strong antimicrobial activity (Grellier *et al.*, 1999). In addition, C. *roseus* contain a very large number of alkaloids about 100 which have been isolated (Verpoorte *et al.*, 1997). Tissue culture technique can be used to produce callus from explants tissue through different plant growth regulators and then to evaluate amounts of alkaloids produced from the callus tissue. Its high commercial value has prompted the production of secondary metabolites through cell culture and cell suspension in recent years (Sato *et al*., 2001).

The present study aimed to determine the optimum culture for callus induction and production of total alkaloids from different ecotypes of Egyptian vinca rosa *(Catharanthus roseus)*.

MATERIALS AND METHODS

Collection of Vinca Rosa Ecotypes

The ecotypes healthy seedlings of uniform size of *Catharanthus roseus* (L.) G. Don were collected from five different Governorate of Egypt (Ismailia, Fayoum, Sharkia, Alexandria and Cairo). Collected seeds were cultivated in greenhouse, faculty of agriculture, Department of Genetics, Zagazig University from November 2013 under natural condition in 25-cm pots that contain five kg of a black soil. The soil was maintained at proper moisture to ensure the optimum growth of the plants. The plant material (leaf and stem) were collected around May 2014. The experiment was conducted according to a simple randomized block design with three replications. The plants were kept free from weeds and irrigated as and when required. Using of leaves and stems of five different ecotypes in tissue culture for callus induction and production of total alkaloids in leaf and stem as well as callus tissues.

Callus induction

Plant materials

Leaf and stem segments from new branches from mature plants seven months old at flowering stage of three different Egyptian ecotypes *C. roseus* (Ismailia, Fayoum and Alexandria) were used for callus induction.

Surface sterilization of explants

Explants were collected, washed thoroughly under running tap water for 15 min, treated in ethyl alcohol 70% for 30 seconds, treated in 1% savlon for five min and were surface sterilized with sodium hypochlorite solution (40%) for 20 min. or different time duration to ensure contamination free culture. The explants were washed three to five times with autoclaved distilled water inside a laminar air flow cabinet.

Establishment of callus cultures

The surface sterilized explants (leaf and stem) were cut into 1.0 - 1.5 cm long segments, stem containing a single node. Explants were individually transferred into 200 ml jars containing 40 ml of MS (Murashige and Skoog, 1962) culture medium for callus production, with 6% (*W/V*) sucrose and different combinations of 2, 4-D (2, 4-dichlorophenoxyacetic acid), BA (Benzyl adenine) and NAA (1-Naphthaleneacetic acid) according to (Kalidass *et al.*, 2009) as follow:

M1= MS + 2, 4-D (1.0 μM) + BA (0.5 μM).

M2= MS + 2, 4-D (0.5 μM) + BA (1.0 μM).

M3= MS + BA (1.0 μM) + NAA (0.5 μM).

M4= MS + BA (0.5 μM) + NAA (1.0 μM).

To test the effect of growth regulators, 24 treatments with factorial combinations of two levels of 2, 4-D, NAA and BA (0.5 and 1 μM) were designed. The pH value of the cultured medium was adjusted to 5.7 ± 0.1 prior to autoclaving (121°C, 15 min) and the medium was solidified with 6-8% agar. The culture conditions were maintained at 25 ± 1 °C under dark condition. The explants were cultured for seven weeks and each treatment was repeated three times. Callus cultures were subculture every 30 days. The callus cultures of the third subculture were used to investigate the effect of 24 different combinations of growth regulators on the growth. All combinations of growth regulators induced callus growth without an organogenesis response over 49 days of cultivation. Tests were done to study the influence of growth regulators on callus production from vinca rosa tissue and to determine the concentration amounts of alkaloids in the produced callus. These tests were done as a factorial experiment in a complete randomized design with three replications.

Determination of Total Alkaloid Content

Collection of the leaf, stem and callus samples

Freshly harvested of *Catharanthus roseus* leaves and stems were collected on January 2014. For determining the extraction of alkaloids from the leaf and stem for ecotypes and the produced callus, a known mass of leaf and stem material of all ecotypes and the callus tissues from the three different ecotype explants (leaf and stem) was finely chopped and subjected to the alkaloid extraction. Total alkaloids content were measured with a spectrophotometer Cary 50 Bio UV-visible (Varian, Italy) associated to a software Cary win UV (Varian, Italy).

Preparation of the leaf, stem and callus samples

The collected and identified plant and callus tissue samples were dried in the sun and finally in a mechanical dryer at 60-70^{0}C. The dried samples were ground to coarse powder with a mechanical grinder and the powdered samples were kept in clean closed glass containers pending extraction. During grinding of sample, the grinder was thoroughly cleaned to avoid contamination with any remnant of previously ground material or other foreign matter deposited on the grinder.

Extraction of the dried powdered samples

About one g of coarse powder was weighted and subjected for extraction with 250 ml methanol using soxhlet apparatus. The process of extraction continues for 24 hours or till the solvent in siphon tube extract become pure or clearly.

Filtration and concentration of the extracts

After extraction process the selected plant extracts were filtered through Whatman© filter paper No.1. The filtrate was collected in a beaker. The plant extracts were concentrated by evaporating the solvent using a rotary evaporator. The residue appeared as a dark brown powder. The dried extract obtained was subjected to phytochemical screening to know the presence of alkaloids and stored in the desiccator and it was used for subsequent experiments. For example to 1 ml of extract added 1 ml of Mayer's reagent and few drop of iodine solution. Formation of yellow colour precipitate indicates the presence of alkaloids.

Preparation of solutions

Bromocresol green (BCG) solution was prepared by heating 69.8 mg bromocresol green with 3 ml of 2N NaOH and 5 ml distilled water until completely dissolved and the solution was diluted to 1000 ml with distilled water. Phosphate buffer solution (pH 4.7) was prepared by adjusting the pH of 2M sodium phosphate (71.6 g Na_2HPO_4 in 1 L distilled water) to pH 4.7 with 0.2M citric acid (42.02 g citric acid in 1 L distilled water). Atropine standard solution was made by dissolving 1mg pure atropine (Sigma Chemical, USA) in 10 ml distilled water.

Preparation of standard curve

Accurately measure aliquots (0.4, 0.6, 0.8, 1 and 1.2 ml) of atropine standard solution and transfer each to different separatory funnels. Then, add 5ml pH 4.7 phosphate buffers and 5 ml BCG solution and shake a mixture with1, 2, 3 and 4 ml of chloroform. The extracts were collected in a 10-ml volumetric flask and then

diluted to adjust volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. Against blank prepared as above but without atropine (Shamsa *et al.*, 2008).

Spectrophotometric assay

Total alkaloid content evaluation was carried out with a spectrophotometric method based on the reaction with BCG (Shamsa *et al.*, 2008), with appropriate changes, A part of extracts residue was dissolved in 2 N HCl and then filtered. One ml of this solution was transferred to a separatory funnel and washed three times with 10 ml chloroform. The pH of this solution was adjusted to neutral with 0.1 N NaOH. Then, 5 ml of pH 4.7 phosphate buffer was added before adding 5 ml of BCG solution and shacked vigorously. Furthermore, the complex formed was extracted with 1, 2, 3, and 4 ml chloroform by vigorous shaking. The extracts were collected in a 10-ml volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470nm.

Estimation of total alkaloids in leaf and stem of plants and callus tissues

For the estimation of total alkaloids in the leaf, stem, and callus tissues, samples in the formulation, suitable aliquots of sample solutions were taken and colour was developed as the method described above. Absorbance of the coloured solution was recorded at 470 nm. The amount of total alkaloids in the plants part samples and callus tissues samples was calculated using calibration curve. The content of the total alkaloids in the different samples was expressed in terms of conessine.

Statistical Analysis

All studied characters for whole plants and callus induction were analyzed by ANOVA with SPSS program version 14 in one way with three or four replications (SPSS, 2009).

RESULTS AND DISCUSSION

Effect of Growth Regulators and Different Ecotypes on Callus Induction and Callus Fresh Weight

Highly significant differences between ecotypes and different growth regulators balance for callus induction frequencies, callus fresh weight and total alkaloid content (Tables 2 and 5). Heritability in broad sense were 20 – 90.7. These result confirm the importance of some ecotypes for alkaloids production and possibility of the selection between different genotypes for improvement of total alkaloid production and cell line for callus production at bioreactor.

The overall response to plant regulators in leaf segments was superior, while stem explants resulted in medium callus induction. Friable greenish or beige callus was successfully induced from wound sites in the young leaf explants (Fig. 1) at a culture time in the range of 19 to 27 days in Fayoum ecotype (Table 1). While stem explants resulted in friable beige, Whitish or compact callus (Fig. 1) at a culture time in the range of 37 to 47 days in Fayoum ecotype (Table 1). Similar results were observed by Singh *et al*. (2011) in *C. roseus.*

Two concentrations of 2, 4-D, BA and NAA (0.5, 1μM) were varied in 24 combinations. All treatments were established with a fresh weight at least 0.3g of callus cultures. Except M3 no callus induction response was noted in the stem explant (Table 3). The stem-derived calluses showed a steady growth with a maximum up to 47 days and decreased till the 60 days (Tables 1 and 3). The lowest percentage of callus induction for stem explant 17% was observed in M4. Table 3 show that the increasing level of BA resulted in a low cell fresh weight. In contrast, NAA in all concentrations had less effect in callus fresh weight enhancing. The highest percentage of callus fresh weight was observed in leaf in M1 (MS + 2, 4-D (1.0 μM) + BA (0.5 μM) but the stem was in M2 (MS + 2, 4-D (0.5 μM) + BA (1.0 μM).

The production of secondary metabolites in callus cultures is controlled by environmental factors, especially different of growth regulators and genotypes (ecotypes). The effects of various concentrations of auxins (2, 4-D, NAA) and cytokinin (BA) on the growth of callus cultures derived from leaf segment and stem explants are presented in Table 4. The maximum fresh weight (3.39 g) of Fayoum ecotype was observed In M1 but the lowest fresh weight (0.3 g) was observed in M3 of Fayoum ecotype too. The interaction of 2, 4-D and BA had a significant effect on cell fresh weight. These results

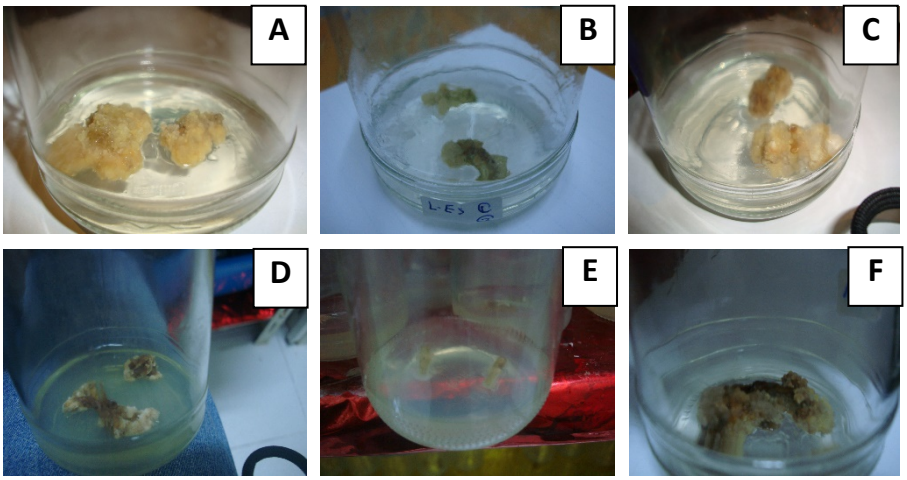

Fig. 1. The effect of 2, 4- D (1.0 µM) and BA (0.5 µM) on callus induction in leaf and stem explants. A. Friable beige stem callus for Ismailia ecotype, B. Friable greenish leaf callus for Ismailia ecotype, C. Stem callus for Fayoum ecotype, D. Leaf callus for Fayoum ecotype, E. No stem callus for Alexandria ecotype and F. Leaf callus for Alexandria ecotype

Table 1. Days to callus induction from leaf and stem explants under dark conditions in different ecotype of Egyptians vinca rosa *(Catharanthus roseus*)

Medium	Fayoum			Ismailia			Alexandria		
MS	Leaf	Stem	Mean	Leaf	Stem	Mean	Leaf	Stem	Mean
M1	19	37	28	21	39	30	21	-	11.5
M2	20	40	30	21	43	32	22	42	32
M3	25	-	12.5	23	-	11.5	21	-	11.5
M4	27	47	37	25	-	12.5	22	-	11
Mean	22.75	31		22.5	20.5		21.5	10.5	

Table 2. Mean sum of squares (MS) for callus induction frequencies (MS1) and callus fresh weight (MS2) for different ecotypes of Egyptian (*Catharanthus roseus)*

SOV	df	Callus induction frequencies		Callus fresh weight	
		MS1		MS2	
		Leaf	Stem	Leaf	Stem
Replications	2	3742.22*	3402.77*	0.5329555	0.072475
Treatments	11	4974.747**	4217.17**	2.19598**	0.142072*
Ecotypes	2	2152.778**	2986.11*	1.90103*	0.375508 *
Media	3	15648.148**	9351.85**	3.692676**	0.145056
Media x Ecotypes	6	578.704	2060.18*	1.5458*	0.062769
Error	24	399.306	599.74	0.4712	0.082686
h^2 in broad sense		32	52	50	20

* and ** significant at 5 and 1% levels respectively. The Means for 3 replications

Table 3. Means of callus induction frequencies (CIF %) of leaf and stem explant on MS medium supplemented with different concentrations and combinations of 2,4-D, BA and NAA(µM) for different ecotypes of Egyptian vinca rosa (*Catharanthus roseus*)

Media code	2,4- D concentration (µM)	BA concentration (µM)	NAA concentration (µM)	CIF mean (%)						
				Leaf			Stem			
				Ism.	Fay.	Alex.	Ism.	Fay.	Alex.	mean
M1	1	0.5	-	100	100	75	50	75	50	75
M2	0.5	1	-	25	100	50	100	83	0	59.6
M3	-	1	0.5	25	83	25	0	0	0	22.2
M4	-	0.5	1	50	25	50	0	17	0	23.6
LSD 0.01					45.63			56.24		
0.05					33.67			41.48		

Ism. = Ismailia, Fay. = Fayoum, Alex. = Alexandria. The Means for 3 replications.

Table 4. Means of callus fresh weight (mg/g) for leaf and stem explant for different media and ecotypes of Egyptian vinca rosa (*Catharanthus roseus*)

	leaf			General mean for callus	stem			General mean for callus
	Ism.	Fay.	Alex.		Ism.	Fay.	Alex.	
M1	0.98	3.39	1.26	1.87	0.5	0.84	0.9	0.75
M2	0.49	1.64	0.88	1.01	0.59	0.6	0.92	0.70
M3	0.49	0.3	0.64	0.47	0.5	0.3	0.64	0.48
M4	0.54	0.32	0.84	0.57	0.51	0.31	0.85	0.55
mean	0.629	1.41	0.90		0.52	0.51	0.82	
General mean		0.98				0.62		
LSD	0.01	1.52668				0.66185		
	0.05	1.123212				0.486944		

Ism. = Ismailia, Fay. = Fayoum, Alex. = Alexandria. M1= MS +BA (0.5) µM + 2, 4-D (1.0) µM, M2= BA (1.0) µM + 2, 4-D (0.5) µM, M3= BA (1.0) µM + NAA (0.5) µM, M4= BA (0.5) µM + NAA (1.0) µM. The Means for 3 replications

demonstrated that, the cytokinins supported growth of callus and provided higher fresh weight. However, auxin, 2, 4-D had more effective on growth of callus when compared to the NAA.

The varying responses *in vitro* culture of *Catharanthus roseus* were noted at different concentrations and combinations for leaf and stem induction calluses. The auxins were found to be the best for callus proliferations and growth. Among auxins, 2, 4-D was the best for increasing callus biomass and total alkaloid content. 2, 4-D was also reported as the most effective auxin in various medicinal plants (Asaka *et al.*, 1993; Misawa, 1994; Junaid *et al.* 2008). While combinations of auxins with cytokinins were found to be better for leaf and stem callus growth and enhancement in alkaloid content.

These results are in accordance with the view of Zenk *et al*. (1977) and Brown (1990) in C.*roseus*, that plant growth regulators have remarkable effects on growth and differentiation and thus metabolism of cultured cells.

Effect of Growth Regulators and Different Ecotypes on Total Alkaloids Content

The crude extract yield obtained from *Catharanthus roseus* extraction requires a specific determination of alkaloids content since the extract contain other chemical compounds different from alkaloids class. Spectrophotometric determination of total alkaloids with BCG is a simple and sensitive method and does not need very specially equipment (Shamsa *et al.*, 2008).

The results of total alkaloid content (TAC) in leaf and stem of all ecotypes are presented in Table 6. TAC was expressed in milligram atropine equivalent (mg CE). The Results of BCG assay showed that the higher mean of TAC was presented in stem extract of Ismailia ecotype (1.88 mg/g) and Fayoum ecotype (1.81mg/g) than leaf extract. While TAC in Cairo ecotype (2.63 mg/g) and Ismailia ecotype (1.92 mg/g) was higher in leaf than stem.

After selection of three ecotypes from Table 7 it was found that the higher mean of alkaloid content was revealed in stem callus extract (1.65 mg/g) than leaf callus extract (1.29 mg/g). While stem callus extract was higher in TAC for Fayoum ecotype (2.22mg/g) and Alexandria ecotype (1.32 mg/g) than leaf callus extract. Fayoum and Ismailia ecotypes was the higher means than original plant.

The results confirmed with the finding of Wong *et al.* (2011) who found that alkaloids content in alkaloid producer species from Apocynaceae family have total alkaloid content major of 1 mg boldine equivalent g−1 (for example *Kopsia fruticosa* leaves that contain 105 μg boldine equivalent g−1; *Nerium oleander* leaves that contain 1400 μg boldine equivalent g−1), demonstrating that *Catharanthus roseus* is a greater source of alkaloids compounds.

In general, we found low cytokinin and higher auxin concentrations to be better for callus proliferation and growth and enhancement of alkaloid content in leaf and stem derived callus of C. *roseus.* The highest enhancement in total alkaloid production resulted from M1 (MS + 2, 4-D (1.0 μM) + BA (0.5 μM), compared with other combinations. Previous data of Verma *et al.* (2012) showed that low auxin and higher cytokinin concentrations to be better for callus proliferation and growth and enhancement of alkaloid content in leaf callus of C. *roseus*. Combinations of 2, 4-D and BA were used by Olivira *et al.* (2001) for enhancement of ramiflorin in callus of *Aspidosperma ramiflorum*, Khan *et al.* (2008) for alkaloid in callus of *Corydylis ophiocarpa*, Yamada and Hashimoto (1982) for tropane alkaloids in callus of *Hyocyamus niger*. The results conflicted with those of Drewes and Staden (1995) for solasodine production in *Solanum mauritianum* and of Rosli *et al.* (2009) for 9-methoxycanthin-6-one production in *Eurycoma longifolia* callus cultures.

Overall, our results showed that M1 medium was the best for biomass production of leaf and stem callus and enhancement of alkaloid accumulation in ecotypes of Egyptian C. *roseus*.

Table 5. Analysis of variance and heritability in broad sense of total alkaloids of different ecotypes of Egyptian vinca rosa *(Catharanthus roseus*)

SOV	Df	MS	
		Leaf	Stem
Replications	3	0.024195	0.0804056 *
Ecotypes	4	0.35583 **	0.3727535 **
Error	12	0.02893308	0.0123283
h^2 in broad sense		79.02	90.7

* and ** significant at 5 and 1% levels respectively. The Means for 4 replications

Table 6. Means of total alkaloids (mg/g DW) for leaf and stem of different ecotypes of Egyptian vinca rosa *(Catharanthus roseus*)

Ecotype	Plant parts		
	Leaf	**Stem**	**Mean**
Ismailia	1.919	1.887	1.9
Fayoum	1.355	1.815	1.58
Alexandria	1.535	1.21	1.37
Cairo	2.063	1.278	1.67
Sharkia	1.521	1.534	1.53
Mean	1.68	1.54	
LSD 0.0١	0.٤٢٤٣	0.٢٧٦٩٥	
0.0٥	0.3026	0.19754	

The Means for 4 replications

Table 7. Means of total alkaloids (mg/g DW) for callus derived from leaf and stem of different ecotypes of Egyptian vinca rosa *(Catharanthus roseus*)

Ecotype	Callus sources		
	Leaf	**Stem**	**Mean**
Ismailia	1.34	1.42	1.38
Fayoum	0.84	2.22	1.53
Alexandria	1.7	1.32	1.51
Mean	1.29	1.65	
LSD 0.01	0.158	0.423	
0.05	0.261	0.255	

Each value is the means of three replicates.

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تأثير منظمات النمو والطرز البيئية لنبات الونكا المصرى على استحداث الكالس وإنتاج القلويدات

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الهدف من البحث دراسة تأثير منظمات النمو وطرز بيئية مختلفة من نبات الونكا روزا المصري مختارة من مناطق الإسماعيلية والفيوم والإسكندرية والشرقية والقاهرة على استحداث الكالس وإنتاج القلويدات، تم توليف منظمات نمو مختلفه ، بيئة إم إس مزودة بأربعة منظمات نمو مختلفه إم ١ (٢ ـ ٤ ـ دى ١ ميكرو مول و بنزيل ادينين (٠.٥) ميكرو مول) ، إم ٢ ((٢-٤ ـ دى ٠.٥ ميكرو مول و بنزيل ادينين ١ ميكرو مول) ، إم ٣ (بنزيل ادنين ١ ميكرو مول ونفثالين اسيتك اسيد ٠.٥ ميكرو مول) و إم ٤ (بنزيل ادنين ٠.٥ ميكرو مول ونفثالين اسيتك اسيد ١ ميكرو مول)، أعطت بيئة إم ١ أعلى قيمه فى نسبة استحداث الكالس(٧٥%) والوزن الطازج (١.٨٧ جم) للكالس الناتج من الورقة، بينما الكالس الناتج من الساق (٠.٧٥ جم) كان الأعلى ايضا من البيئات الأخرى، تم حساب المحتوى الكلى للقلويدات باستخدام الاسبكتروفوتوميتر مع صبغة أخضر البروموكريزول . أظهرت النتائج أن المحتوى الكلى للقلويدات تفوق فى الكالس الناتج من الساق عن كلا من الكالس الناتج من الورقة وساق وورقة النبات الأصلى . من النتائج الهامه ان الطراز البيئى الفيوم امتلك أعلى محتوى من القلويدات (٢.٢٢ ملجم/جرام) للكالس الناتج من الساق عن كل الطرز البيئية تحت الدراسة

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