

Successful Application for Enhancement and Production of Anthocyanin Pigment from Calli Cultures of Some Ornamental Plants

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Abstract: An efficient and promising protocol for achievement and enhancement of anthocyanin production from calli cultures of some ornamental plants was established. The effect of different concentrations of 2,4-D and BAp or NAA and Kin addition to MS-media on callus production from internode's explants of *Catharanthus roseus*, *Celosia argentea* and *Cordyline terminalis* was investigated. The highest value of calli growth dynamics i.e., fresh, dry weights (gm) and dry matter content (%) were recorded with catharanthus, celosia and cordyline explants, respectively. Moreover, the maximum value of calli production was recorded with MS-medium supplemented with 1.0 mg/l 2,4-D + 3.0 mg/l BAp. The effect of L-phenylalanine in combinations with Ca⁺⁺ at the concentrations of 1, 3, 5 and 10 µM and 0.25, 0.5, 0.75 and 1.0 µM, respectively on enhancement of total anthocyanin production were studied. The optimum supplementations of L-phenylalanine and Ca⁺⁺ to modify MS-medium were 3 and 0.5 µM, respectively. The highest values of total anthocyanin production 197.98, 164.32 and 78.73 µg/gm were recorded with celosia, cordyline and catharanthus calli cultures, respectively.

Key words: anthocyanin, callus, catharanthus, celosia, cordyline, phenylalanine and Ca⁺⁺

INTRODUCTION

In recent advances of plant biotechnology techniques, there have been a number of reports on the production of secondary metabolites such as alkaloids, quinones, terpenes, and flavonoids in plant tissue culture (Mori, *et al.*, 1994). Anthocyanin pigments and derivatives are flavonoid and unique to the plant kingdom. They are expected to be beneficial to human health as potential anti-cancer (Castonguay, *et al.*, 1997); cardio protective (Morazzoni and Bombardelli 1996); anti-inflammatory; and as antioxidant properties (Liu, *et al.*, 2002). Therefore, anthocyanin has also been produced in various plants by tissue culture: wild carrot (Dougall, and Weyrauch 1980), *Hibiscus sabariffa* (Mizukami, *et al.*, 1988), *Vitis* sp. (Tamura, *et al.*, 1989) and strawberry (El-Sawy and Taha 2000).

Catharanthus roseus (L.) G. Don (Apocynaceae) is one of the most important medicinal plants. In the past few decades a large number of publications have covered the improving knowledge on the antitumour alkaloids of catharanthus. Many reports in the literature detail the effects of modifications to culture conditions upon the yield of valuable secondary metabolites in cultured cells (van der Heijden *et al.*, 2004). On the contrary, the anthocyanin pigments productions *in vivo* and *in vitro* of this species have attracted little attention. The first work on anthocyanin production in callus cultures of *C. roseus* was reported by (Carew and Krueger 1976). Furthermore, (Knobloch *et al.*, 1982) observed that after transfer of cell suspension cultures of *C. roseus*, grown in Murashige and Skoog (1962) (MS) medium containing 2.6 M of 2,4-D, into a 10-fold volume of an 8% aqueous sucrose solution and upon continuous irradiation with fluorescent lamps, the cultures turned red due to the formation of anthocyanin. On other hand, (Godoy-Hernandez and Loyola-Vargas 1997) reported that, total anthocyanin was achievement by addition of 20 mM of acetylsalicylic acid as a precursors to *C. roseus* cell line medium to 1476 % of the control cultures. Moreover, (Ohlsson and Berglund 2001) they reported that the addition of GA₃ to *C. roseus* cell cultures was enhanced of glutathione reeducates activity to a maximal activity of 135% of the control and the content of anthocyanin was increased about 137% of the control. Furthermore, in a successive study (Filippini, *et al.*, 2003) obtained a selected cell strain, of *C. roseus* having high and stable anthocyanin production.

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Cordyline terminalis (L.) Kunth. (Agavaceae) with its beautiful and attractive red decorative foliage is one of the most economically important ornamental houseplant. Concerning callus production from *C. terminalis* there are only a few reports on *in vitro* clone propagation through callus cultures were recorded by (Mee, 1978; Khan and Saeed 2001; Ray, *et al.*, 2006). However, there are no available publications about anthocyanin production from callus cultures of this plant. May be this is a first study about anthocyanin production from this ornamental plant.

Celosia argentea (L.) cristata (Amaranthaceae). and its uses as antibacterial; astringent; haemostatic; hypertensive; ophthalmic and parasitic (Kunkel, 1984). and up to now limited work has been done on the plant tissue culture of this family. However, callus production and adventitious shoots formation from different plants belong this family have been reported by (Flores *et al.*, 1982; Bagga *et al.*, 1987). Also, may be this is a first investigation about anthocyanin production from callus cultures of *Celosia argentea*.

L-Phenylalanine is an initial compound of anthocyanin biosynthesis in the phenylpropanoid and flavonoid metabolic pathways of plant cells. Compared to other precursors in the pathways, L-Phenylalanine is relatively inexpensive and more effective for anthocyanin accumulation (Edahiro *et al.*, 2005).

Calcium is an essential element for either plant or animal certain enzymes formation. Calcium is also a known retardant of senescence (Knobloch *et al.*, 1982) and has been found to stimulate amino acid transport at millimolar concentrations (Sudha and Ravishankar 2003).

The objectives of this study were to clarify the relationship between callus growth and anthocyanin production from intrnod explants of *Catharanthus roseus*, *Celosia argentea* and *Cordyline terminalis*. Futhermore, study the effect of L-Phenylalanine and Calcium feeding as a precursors on anthocyanin accumulation and production.

MATERIALS AND METHODS

Plant Materials:

Internode's explants were excised from sterilized *Catharanthus roseus*, *Celosia argentea* and *Cordyline terminalis* plantlets which were washed several times by tap water and little drops of Tween 20, then, sterilized by soaking for 30 sec, in 70 % Ethanol, followed by immersion in a 25 % of commercial Clorox solution (0.25 % NaOCl) for 10 min. Then they were rinsed three times with sterile distilled water. Three explants from each type were transferred into 200 ml jar containing 25 ml sterile medium (MS) with 3 % sucrose, 0.7 agar. The pH of the medium was adjusted to 5.8 using 0.1N KOH or 0.1N NaOH prior autoclaving at 121°C for 20 min. The obvious explants were subsequently cultured on MS-Medium supplemented with different concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D), N⁶-benzylaminopurine (BAP), 1-naphthalen acetic acid (NAA) and 6-furfurylaminopurine(Kin) as the following:

- MS + 1 mg/l 2,4-D + 1mg/l Kin.
- MS + 1 mg/l 2,4-D + 3mg/l Kin.
- MS + 1 mg/l 2,4-D + 5mg/l Kin.
- MS + 1 mg/l 2,4-D + 1mg/l BAP.
- MS + 1 mg/l 2,4-D + 3mg/l BAP.
- MS + 1 mg/l 2,4-D + 5mg/l BAP.
- MS + 1 mg/l NAA + 1mg/l Kin.
- MS + 1 mg/l NAA + 3mg/l Kin.
- MS + 1 mg/l NAA + 5mg/l Kin.
- MS + 1 mg/l NAA + 1mg/l BAP.
- MS + 1 mg/l NAA + 3mg/l BAP.
- MS + 1 mg/l NAA + 5mg/l BAP.

All cultures were incubated under the temperature of 26 ±1 °C under 16 hr of fluorescent lighting lamps 1300 Lux per day and 8 hr darkness cycle. For mass callus production from different type's cultures, subcultures were done every 4 weeks for 3 months.

Determination of Callus Growth Parameters:

Growth patterns of callus production from different types of all cultures were determined after 30 days from incubation under light condition as follow:

- Percentage of callus formation (%).
- Fresh weight (gm/jar).
- Dry weight (mg/jar).
- Percentage of dry matter content (%).
- Growth dynamics during 5 weeks of subculturing (gm/jar).

Effect of L-Phenylalanine and Ca⁺⁺ Feeding to Media as a Precursors on Anthocyanin Synthesis and Production:

About 250 (mg/jar) of callus fresh weight derived from different type of cultures were inoculated into 25 ml of fresh MS-medium containing 1 mg/l 2,4-D + 3 mg/l BAp. The combinations of filtrated sterilized L-phenylalanine and calcium chloride (Ca⁺⁺) at the concentrations 0, 1, 3, 5 and 10 µM and 0, 0.25, 0.5, 0.75 and 1.0 µM, respectively were added to culture media. The total anthocyanin was recorded after 5 weeks of inoculation.

Determination of Anthocyanin (µg/gm) in Callus Fresh Weight:

Half gram of fresh calli was weighted in a 15 ml plastic centrifuge tube and broken to small pieces using a forceps. Five ml of methanol containing 1% concentrated HCl at 4°C was added to sample. After vortex, the samples were then centrifuged at 1000 X gm for 10 min. absorbency of the clear supernatant was measured at 528 nm. Anthocyanin content was calculated with the extinction coefficients:

(E1^{1%} cm = 680 at 528 nm) obtained by purified peonidin 3-glucoside according the described method by (Mori *et al*, 1993). The major anthocyanin had been previously identified. Total anthocyanin yield was expressed as (mg/gm) callus fresh weight.

RESULTS AND DISCUSSIONS

Results:

Callus Production:

Callus Formation (%):

Internode's explants of either *Catharanthus roseus*, *Celosia argentea* and *Cordyline terminalis* were cultured on MS-medium supplemented with 1 mg/2,4-D in combination with 1 or 3 or 5 (mg/l) of either Kin or BAp. As well as the internodes explants of those plants were cultured on MS-medium supplemented with NAA in combination with 1 or 3 or 5 (mg/l) of either kin or BAp. Data present in Table (1) shows the effect of MS-medium supplemented with different concentrations of previous auxins and cytokinens on percentage of callus formation. The maximum percentages of callus formation (100, 85 and 67 %) were recorded with catharanthus, celosia and cordyline, respectively. Furthermore, MS-medium supplemented with 1 mg/l 2,4-D and 3 mg/l BAp gave the highest value of callus formation from internode's explants of those plants as compared with other supplementation.

Table 1: Effect of MS-medium supplemented with 2,4-D, NAA, Kin and BAp on percentage of callus formation derived from *Catharanthus*, *Celosia* and *Cordyline* internode explants under light condition.

MS-supplemented with:	Internodes explants of:		
	<i>Catharanthus roseus</i>	<i>Celosia argentea</i>	<i>Cordyline terminalis</i>
Free growth regulators	---	---	---
1 mg/l 2,4-D + 1 mg/l Kin	60	42	21
1 mg/l 2,4-D + 3 mg/l Kin	70	45	25
1 mg/l 2,4-D + 5 mg/l Kin	63	43	23
1 mg/l 2,4-D + 1 mg/l BAp	97	74	53
1 mg/l 2,4-D + 3mg/l BAp	100	85	67
1 mg/l 2,4-D +5 mg/l BAp	98	80	60
1 mg/l NAA+ 1 mg/l Kin	75	57	28
1 mg/l NAA + 3 mg/l Kin	85	59	35
1 mg/l NAA + 5 mg/l Kin	81	55	30
1 mg/l NAA + 1 mg/l BAp	89	61	39
1 mg/l NAA + 3mg/l BAp	95	69	49
1 mg/l NAA +5 mg/l BAp	93	65	45

Each treatment is the average of 5 replicates.

Callus Fresh Weight (gm/jar):

Data tabulated in Table (2) shows that the maximum value of callus production as fresh weight (gm/jar) was recorded with catharanthus as a compared with celosia and cordyline, respectively. The best supplementations from auxins and/or cytokinens to MS-medium for highest value of mass callus production were 2,4-D as auxin and BAp as cytokinens as compared with either 2,4-D with Kin or NAA with Kin or BAp. Furthermore, the optimum concentrations from 2,4-D and BAp were 1 and 3 mg/l, respectively as compared with other concentrations. The descending orders of callus fresh weight (gm/ jar) were 1.85, 1.25 and 0.68 in catharanthus, celosia and cordyline, respectively. Whereas the minimum value of callus fresh weights 0.51, 0.25 and 0.15 (gm/jar) were recorded with catharanthus, celosia and cordyline respectively, when MS-medium was supplemented with 1 mg/l from each of 2,4-D and Kin.

Table 2: Effect of MS-medium supplemented with 2,4-D, NAA, Kin and BAp on callus fresh weight (gm/jar) derived from of *Catharanthus*, *Celosia* and *Cordyline* internode explants under light condition.

MS-supplemented with:	Internodes explants of:		
	<i>Catharanthus roseus</i>	<i>Celosia argentea</i>	<i>Cordyline terminalis</i>
Free growth regulators	---	---	---
1 mg/l 2,4-D + 1 mg/l Kin	0.51 ± 0.044	0.25 ± 0.025	0.15 ± 0.008
1 mg/l 2,4-D + 3 mg/l Kin	0.65 ± 0.054	0.35 ± 0.017	0.21 ± 0.005
1 mg/l 2,4-D + 5 mg/l Kin	0.58 ± 0.085	0.29 ± 0.013	0.18 ± 0.001
1 mg/l 2,4-D + 1 mg/l BAp	0.95 ± 0.025	0.85 ± 0.035	0.58 ± 0.035
1 mg/l 2,4-D + 3mg/l BAp	1.85 ± 0.123	1.25 ± 0.145	0.68 ± 0.075
1 mg/l 2,4-D +5 mg/l BAp	1.25 ± 0.154	1.15 ± 0.127	0.65 ± 0.056
1 mg/l NAA+ 1 mg/l Kin	0.68 ± 0.058	0.40 ± 0.053	0.25 ± 0.017
1 mg/l NAA + 3 mg/l Kin	0.74 ± 0.035	0.51 ± 0.027	0.35 ± 0.029
1 mg/l NAA + 5 mg/l Kin	0.72 ± 0.087	0.45 ± 0.089	0.31 ± 0.023
1 mg/l NAA + 1 mg/l BAp	0.75 ± 0.012	0.54 ± 0.043	0.38 ± 0.025
1 mg/l NAA + 3mg/l BAp	0.85 ± 0.093	0.73 ± 0.029	0.50 ± 0.043
1 mg/l NAA +5 mg/l BAp	0.78 ± 0.025	0.68 ± 0.054	0.43 ± 0.039

Each treatment is the average of 5 replicates. ± Standard error

Callus Dry Weight (gm/jar):

Dry weight was determined as indicate for true growth pattern of callus production. Table (3) shows the optimum supplementation of BAp was found to be 3.0 (mg/l) and 1 mg/l of 2,4-D to MS-medium recorded the highest value of callus dry weight. The best results of dry weight 0.093, 0.085 and 0.054 (gm/jar) were estimated with catharanthus, celosia and cordyline, respectively. As well as the lowest dry weights were recorded with MS-medium which supplemented with 1 mg/l from each of 2,4-D and Kin.

Table 3: Effect of MS-medium supplemented with 2,4-D, NAA, Kin and BAp on callus dry weight (gm/jar) derived from of *Catharanthus*, *Celosia* and *Cordyline* internode explants under light condition.

MS-supplemented with:	Internodes explants of:		
	<i>Catharanthus roseus</i>	<i>Celosia argentea</i>	<i>Cordyline terminalis</i>
Free growth regulators	---	---	---
1 mg/l 2,4-D + 1 mg/l Kin	0.038 ± 0.002	0.017 ± 0.005	0.008 ± 0.005
1 mg/l 2,4-D + 3 mg/l Kin	0.049 ± 0.003	0.025 ± 0.003	0.011 ± 0.004
1 mg/l 2,4-D + 5 mg/l Kin	0.043 ± 0.005	0.015 ± 0.006	0.009 ± 0.0001
1 mg/l 2,4-D + 1 mg/l BAp	0.078 ± 0.003	0.066 ± 0.005	0.047 ± 0.008
1 mg/l 2,4-D + 3mg/l BAp	0.153 ± 0.017	0.085 ± 0.007	0.054 ± 0.007
1 mg/l 2,4-D +5 mg/l BAp	0.096 ± 0.014	0.075 ± 0.008	0.050 ± 0.003
1 mg/l NAA+ 1 mg/l Kin	0.058 ± 0.025	0.038 ± 0.005	0.022 ± 0.008
1 mg/l NAA + 3 mg/l Kin	0.073 ± 0.035	0.031 ± 0.007	0.019 ± 0.005
1 mg/l NAA + 5 mg/l Kin	0.065 ± 0.022	0.032 ± 0.004	0.029 ± 0.006
1 mg/l NAA + 1 mg/l BAp	0.066 ± 0.031	0.049 ± 0.003	0.035 ± 0.009
1 mg/l NAA + 3mg/l BAp	0.071 ± 0.024	0.062 ± 0.005	0.045 ± 0.005
1 mg/l NAA +5 mg/l BAp	0.068 ± 0.031	0.055 ± 0.008	0.039 ± 0.008

Each treatment is the average of 5 replicates. ± Standard error

Callus Dry Matter Content (%):

The maximum values of dry matter content 9.86 %, 9.50 % and 9.35% were recorded with catharanthus, celosia and cordyline, respectively. MS medium supplemented with 1 mg/l NAA + 3 mg/l Kin in case of *Catharanthus roseus*. However, MS medium supplemented with 1 mg/l each of NAA and Kin gave the highest record of dry matter content for *Celosia argentea*. Furthermore, the supplementation of MS-medium with 1 mg/l NAA + 5 mg/l Kin revealed the highest percentage of dry matter content (%) of derived calli from *cordyline terminalis* 9.35% . whereas, the minimum value of dry matter content (5%) was recorded with derived calli from cordyline explants as compared with other calli types Table (4).

Table 4: Effect of MS-medium supplemented with 2,4-D, NAA, Kin and BAp on percentage of callus dry matter content (%) derived from of *Catharanthus*, *Celosia* and *Cordyline* internode explants under light condition.

MS-supplemented with:	Internodes explants of:		
	<i>Catharanthus roseus</i>	<i>Celosia argentea</i>	<i>Cordyline terminalis</i>
Free growth regulators	---	---	---
1 mg/l 2,4-D + 1 mg/l Kin	7.451	6.8	5.33
1 mg/l 2,4-D + 3 mg/l Kin	7.51	7.14	5.24
1 mg/l 2,4-D + 5 mg/l Kin	7.41	5.17	5.0
1 mg/l 2,4-D + 1 mg/l BAp	8.21	7.76	8.10
1 mg/l 2,4-D + 3mg/l BAp	8.27	6.8	7.94
1 mg/l 2,4-D +5 mg/l BAp	7.68	6.53	7.69
1 mg/l NAA+ 1 mg/l Kin	8.53	9.5	8.8
1 mg/l NAA + 3 mg/l Kin	9.86	6.08	5.43
1 mg/l NAA + 5 mg/l Kin	9.03	7.11	9.35
1 mg/l NAA + 1 mg/l BAp	8.8	9.07	9.21
1 mg/l NAA + 3mg/l BAp	8.35	8.49	9.0
1 mg/l NAA +5 mg/l BAp	8.72	8.09	9.06

Each treatment is the average of 5 replicates

Callus Growth Dynamics During Five Weeks of Cultivation:

An equal weight ~250 (mg/jar) from obtained callus of catharanthus, celosia and cordyline were subcultured on freshly Ms-medium supplemented with 1 mg/l 2, 4-D and 3 mg/l BAp. Callus fresh weights were determined weekly during five weeks of cultivation. Datin Fig. (1) illustrated that , the maximum values of callus as fresh weight 1.458, 0.638 and 0.463 (gm/jar) were recorded with *Catharanthus roseus* Fig. (2-A), *Celosia argentea* Fig. (2-B) and *Cordyline terminalis* (Fig. 2-C), respectively. The 3rd week of cultivation showed the best results of freshly mass calls production as compared with other weeks which were decreased in either 4th or 5th week of cultivation.

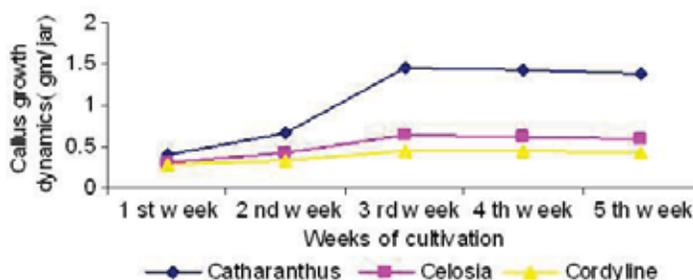


Fig. 1: Calli growth dynamics of *Catharanthus roseus*, *Celosia argentea* and *Cordyline terminalis* during five weeks of cultivation.

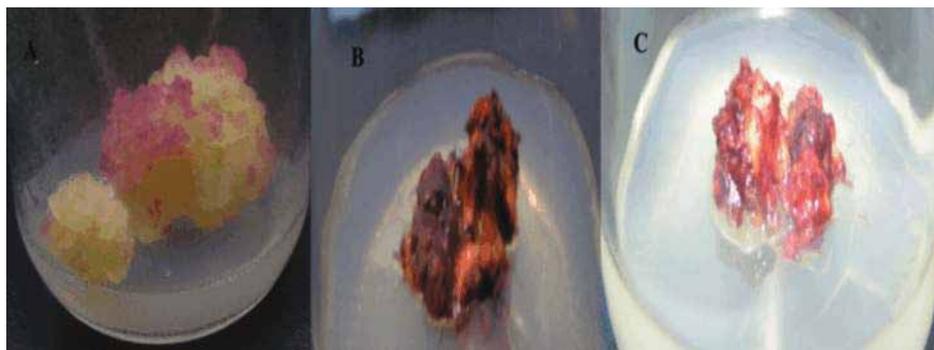


Fig. 2: Callus production from *Catharanthus roseus* (A), *Celosia argentea* (B) and *Cordyline terminalis* (C).

Anthocyanin Determination (µg/gm) Callus Fresh Weight:

The effect of different concentrations 0, 1, 3, 5 and 10 µM of L-phenylalanine and calcium chloride (Ca⁺⁺) at the concentrations 0, 0.25, 0.5, 0.75 and 1.0 µM on total anthocyanin content (µg/gm callus fresh

weight) was investigated. Presents data in Figs (3-A, B and C) shows that the highest values of total anthocyanin accumulation 197.98, 164.32 and 78.73 $\mu\text{g}/\text{gm}$ callus fresh weight were recorded with *Celosia argentea* (Fig.3-A) *Cordyline terminalis* (Fig. 3-B) and *Catharanthus roseus* (Fig.3-C), respectively. Furthermore, the optimum supplementation of MS-medium with 1 mg/l 2, 4-D and 3 mg/l BAp (the best medium for either callus and/or anthocyanin production) with 0.5 μM of calcium chloride (Ca^{++}) in combination with 3.0 μM of L-phenylalanine shows the best concentration from both precursors on enrichment and enhancement of total anthocyanin accumulation and production in different study plants.

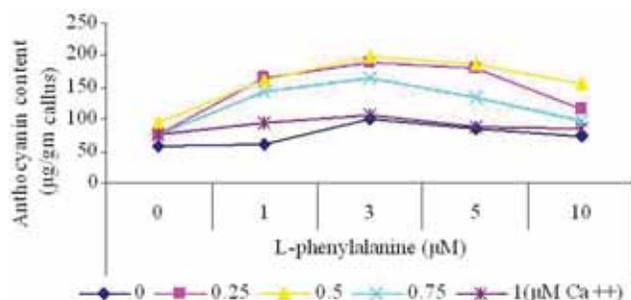


Fig. 3-A: Effect of different combinations of L-phenylalanine (μM) and Ca^{++} (μM) on total anthocyanin content ($\mu\text{g}/\text{gm}$) on callus of *Celosia argentea*.

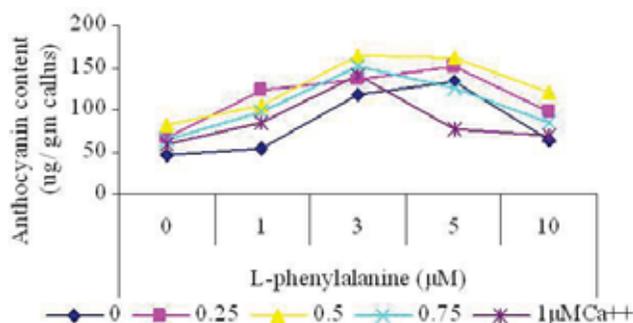


Fig. 3-B: Effect of different combinations of L-phenylalanine (μM) and Ca^{++} (μM) on total anthocyanin content ($\mu\text{g}/\text{gm}$) on callus of *Cordyline terminalis*.

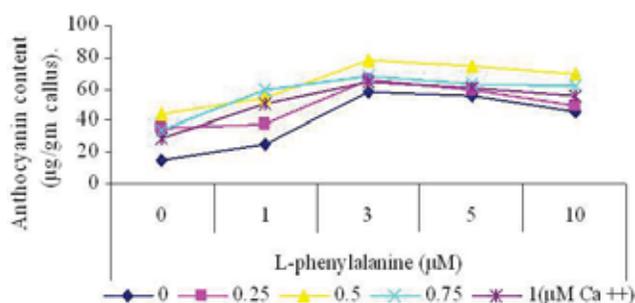


Fig. 3-C: Effect of different combinations of L-phenylalanine (μM) and Ca^{++} (μM) on total anthocyanin content ($\mu\text{g}/\text{gm}$) on callus of *Catharanthus roseus*.

Discussion:

The effect of different concentrations from 2, 4 –D and / or NAA as auxins in complementation with BAp and /or Kin as cytokinins on callus and/ or anthocyanin production from internode’s explants of *Catharanthus roseus*, *Celosia argentea* and *Cordyline terminalis* were investigated. On this respect ⁽¹⁾ reported that the best results of callus production was obtained when the leaf explants of *Fragaria ananassa cu.* Skikinari strawberry cultivar were cultured on MS medium containing 1 mg/l 2,4–D + 1 mg/l BAp. However, the differences in

our results and obtained results by (Mori *et al.*, 1994) may be due to the variation of plants. Furthermore, they investigated the different concentrations of 2,4-D and BAP on anthocyanin accumulation on above cultivar, they reported that the optimum concentrations of 2, 4-D was (1 mg/l) and BAP ranged from (0.0 to 0.1 mg/l). Concerning the optimum concentration of 2,4-D this result were in close with our obtained result. However, concerning BAP concentration it was found that the concentration of BAP (3 mg/l) more suitable for anthocyanin production with investigated plants. In this direction, (Binns, 1994) reported that cytokinins are important regulators of many aspects of plant development, including cell division, nutrient mobilization, senescence, chloroplast development, and apical dominance. Despite the widely acknowledged importance of cytokinin in plant development, very little is known about its mechanism of action at the molecular level. Cytokinins have been shown to affect the expression of specific genes by both increasing and decreasing the abundance of particular proteins or mRNAs.

Furthermore, in agreement with our obtained results, (Fang *et al.*, 1998) reported that BAP had a powerful and overriding influence on both of cell growth cycle and total anthocyanin production of wild *Vaccinium*. However, on contrast of obtained results, (Meyer and Van Staden, 1995). reported that BAP stimulated anthocyanin production, but depressed cell growth in callus cultures of *Oxalis lineasis*. One response many plants have to cytokinin treatment is to accumulate anthocyanins. Cytokinins have been shown to cause an increase in anthocyanin accumulation in tissue culture and in parts of intact plants. For example, anthocyanin accumulation in response to cytokinins was shown in carrot (*Daucus carota*) suspension culture cells (Ozeki and Komamine, 1985). The differences in anthocyanin content may be due to the structural genes encoding the enzymes of the anthocyanin biosynthetic pathway are conserved among different plant species (Holton and Cornish, 1995). and their expression is regulated by several regulatory genes (Quattrocchio *et al.*, 1993). On other hand, (Mori and Sakurai 1994; Suzuki, 1995; Zhong and Yoshida 1995). reported that, the suitable phytohormones source can exert a profound effect on both of callus production and anthocyanins accumulation in different types of some plants.

Concerning the effect of L-phenylalanine on anthocyanin production. (Piovan and Raffaella, 2007). reported that in *Catharanthus roseus* cell cultures the accumulation of anthocyanins are dependent upon the environmental conditions (phosphate, mineral nitrogen, L-phenylalanine, trans-cinnamic acid, sucrose) and that light is an essential stimulus. On other hand, (Kakegawa *et al.*, 1995). reported that a high level of endogenous L-Phenylalanine following the cessation of cell division triggers the induction of anthocyanin biosynthesis in *Vitis* cell culture and, it was suggested that the intracellular L-Phenylalanine accumulated at high levels is used as a biosynthetic precursor material for anthocyanin and related flavonoids, and at the same time, has a function as a kind of signal that promotes the transcription of the genes on the anthocyanin biosynthetic pathway. This results in agreement with our obtained results concerning the supplementation of MS-medium with high level of L-phenylalanine.

Whilst, concerning the influence of Ca⁺⁺ on anthocyanin accumulation was studied by (Sudha and Ravishankar, 2003). They reported that, the treatment of *Daucus carota* cell cultures with low levels of calcium resulted in the enhancement of both growth and anthocyanin production. This results due to stimulate of putrescine and spermidine uptake, whereas higher levels were found to be inhibitory for putrescine uptake in carrot cell cultures. Also, they reported that Ca²⁺ reversed the inhibitory effect of polyamines on ethylene production, partly or in full, and that Ca²⁺ supplied together with polyamines diminished their action, indicating probable involvement of an initial ionic attachment mechanism. This results were in line with our obtained results, because calcium as an essential element both animals and plants and it is also a know as a retardant of senescence (Lieberman and Wang, 1982).

Conclusion:

From the obtained results, it could be concluded that culturing of internode's explants of *Celosia argentea* on MS-medium supplemented by 1.0 mg/l 2,4-D in complement with 3.0 mg/l BAP and the addition of L-phenylalanine and Ca⁺⁺ at the level of 3.0 and 0.5µM respectively gave the best results of anthocyanin production as compared with *Cordyline terminalis* and *Catharanthus roseus* respectively.

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