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Title: Production of anthocyanin in cell suspension culture of grape (*Vitis vinifera* L.)

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Abstract:
Background: *Anthocyanin plays an important role as an anti-cancer, antioxidant in human health, it prevents heart attacks, and strengthens the immune system. Cell suspension culture is an effective, safe, and environmentally friendly method for the production of in vitro anthocyanin from grapes Vitis vinifera cv. Crimson grown in the Egyptian desert.*
Material and Methods: *In this study, the callus from leaf explants was initiated on Gamborg medium (B5) containing different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and N6-furfuryladenine (kinetin; kin). B5 medium supplemented with 9.1 µM 2,4-D and 0.93 µM kin gave the best callus induction and proliferation. Salicylic acid (SA) as an elicitor and L-phenylalanine (L-Phe) as a precursor were used to improve the productivity of anthocyanin from V. vinifera cell suspension cultures.*
Results: *It was found that both SA and L-Phe could promote the accumulation of anthocyanin in suspended grape cells. The highest biomass of callus was reached when the precursor L-Phe was added to the medium at 302.7 µM. In this regard, after eight weeks of culture, L-Phe increased the biomass of anthocyanin in the callus of V.vinifera by 5.03 times compared to the intact plant. After 15 days of incubation on shake-flask with the addition of L-Phe at 302.7 µM has been obtained the highest synthesis of anthocyanin where reached 31.037 fold compared with the intact plant. The shake-flask culture with the use of SA or Phe, is a promising method for the anthocyanin production from V. vinifera cv. Crimson, and is a feasible technique that is supported by industrial and academic interests.*
Key Words:*. Crimson; Shake- flask culture; Plant cell culture; Salicylic acid; phenylalanine.*

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I. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most cultivated fruit plants and an economically essential crop worldwide [1]. Grapes were consumed either as fresh fruit or as products derived from them [2,3]. Therefore, it plays an important role in economy of countries [4]. *V. vinifera* fruit contains various phenolic compounds, flavonoids and rich sources of anthocyanins [5]. Anthocyanins are water-soluble natural pigments, according to its extraction from grapes and, it was found out that anthocyanin contain a range of colours starting from pink through red, violet and dark blue. [6, 7]. So, they are considered as natural pigments to replace the synthetic colourants in many kinds of foods it has brilliant colour pigmentation [8]. They are not only used for food and drink additives to obtain attractive natural colouration, but also have for importance role in production pharmaceutical and cosmetic products. Therefore, anthocyanins are seen as multifunctional secondary metabolites of particular interest [9,10]. In addition to, anthocyanin has an important role in human health, It is used to enhance vascular elasticity, prevent cardiovascular disease, and protect the liver from damage [11, 12]. They function as natural antioxidants, anticarcinogenic properties, anti-inflammatory, antiallergic, antiulcer, immune enhancing and antibiotic further more it has a wide range of beneficial biological activities and properties and many applications in medicnal and industry [13, 14]. Extraction of anthocyanins from the whole plant by conventional methods is a risky process, which is a lack in purity and the amount of active substance, depending on the season and the consumption of a large number of plants. Therefore, it wise to used tissue culture and cell suspension cultures for production of anthocyanins [15]. The production of phytochemicals by plant cultures *in vitro* has become commonly used and considered an important application. [16]. *In vitro* plant cell and tissue culture is to allow, the continuous production of active components indepentent of geographical or climatic conditions and within a short time period as compared to the extracted from plants.[17]. In addition, cells are free of diseases and are avoid seasonal variations, and it is possible finding products not yet found in nature [18]. Many researchers focused on stimulating the production of

secondary compounds from the cell suspension culture from the plant and also worked on increasing them through selecting the best media [19] and choosing the optimum explant for tissue culture [20, 21], elicitation [22, 23, 24] and addition of precursors [25]. Accumulation of anthocyanin in callus and suspension cultures is very important on the large scale, but also with the investigation of various factors influencing on anthocyanin production such as the precursor and elicitors [26]. From previous studies, some authors worked on stimulation of anthocyanin synthesis in *V. vinifera* cell suspension culture [27, 28, 29,30]. A well-known case of this concept is the addition of salicylic acid (SA) and phenylalanine (L-Phe), to enhance the productivity of anthocyanin [28]. Morever, the use of *in vitro* technology has allowed the production of anthocyanins, large scale production of these pigments in standardized conditions remains as one of the great challenges for researchers in plant biotechnology field [31]. For the best of my knowledge, this study is considered the first in the production of anthocyanin from suspended cultures for the grape cv. Crimson grown in Egypt. The main aim of this work was to produce and increase the total anthocyanin content (TAC) from cells in shake-flask culture of *V. vinifera* cv. Crimson, which was cultivated in the Egyptian desert through investigating the effect of SA as elicitor and L-Phe as precursor.

II. Material and Methods

2.1 Chemicals

All chemical reagents Sigma-Aldrich (St. Louis, MO, USA).

2.2 Plant material

Leaves of *Vitis vinifera* L.cv. Crimson (Red cultivar) of 6 years-old trees grown in a farm on the road to Cairo Alexandria Desert in Egypt were used as explants.

2.3 Callus induction and prolefration

Explants of *V. vinifera* were carried out by rinsing with running tap water with the use of detergent for two hours to get rid of dust. Surface sterilization was carried out using dipping with an ethyl alcohol concentration of 70% for one minute followed by 2.5% sodium hypochlorite for 15 minutes and then wash five times with sterile distilled water under laminar flow hood cabinet (Holten Laminar Air HVR 2448, USA) . Young leaves cut into squares in the size 1 cm× 1cm were cultured on the surface of Gamborg (B5) basal medium [32] supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (4.55, 9.1, and 18.2, µM) and N6-furfuryladenine (Kinetin; kin) (0.93 µM). B5 medium without growth regulators was used as a control. The medium was supplied with 3% w/v sucrose and 100 mg/l myo-inositol. The pH of the medium was adjusted to5.7-5.8 and solidified with 2.75 g/l phytagel. Nutrient medium (45 ml volumes) were dispensed into 350 ml glass jars. Then were sealed with autoclavable polypropylene caps and autoclaved at 121°C under 1.06 kg/cm2 for 20 minutes (Harvy Sterlemax autoclave, Thermo Scientific, USA). Cultures were maintained under darkness for four weeks, after that transferred to a 16-hour photoperiod provided by cool-white fluorescent tubes (F140t9d/38, Toshiba) at a temperature of 25 ± 2ºC and callus morphology were recorded after eight weeks of culture. Callus proliferation was carried out using different concentrations of 2,4-D (4.55, 9.1, 18.2µM) and kin (0.93 µM). The fresh weight (FW) of callus (g/jar) and percentage of its increase were recorded after six weeks. Callus cultures were transferred and subcultured to fresh medium every four weeks for proliferation.

2.4 Elicitor and precursor feeding

Due to the superiority of 2,4-D at 9.1 µM plus kin at 0.93 µM concentration, 5.0 g of callus fresh weight /jar of this treatment was taken and established on B5 solid medium with concentrations of181, 362, 724 µM salicylic acid (SA) as the elicitor and 302.7, 605.4, and 908 µM L-phenylalanine (L-Phe) as precursor. The control treatment is the same medium without elicitor and precursor. After harvesting callus FW, dry weight (DW), percentage of increase in FW of callus and anthocyanin content (µg/g Dry weight), were measured after four weeks of culture. DW was determined after drying at the callus at 70ºC in an oven until a constant weight was obtained.

2.5 Suspension cultures

Suspension cultures were initiated using 5.0 g of fresh friable callus formed from in the best proliferation B5 liquid medium (9.1 µM 2,4-D + 0.93 µM kin + 302.7 µM L-Phe) into 250 ml Erlenmeyer flasks containing 50 ml medium. The pH of the medium was adjusted 5.7-5.8 before autoclaving as mentioned above. Flasks were closed with cotton plugs and two layers of alumonium foil and incubated at 27±2 ºC with continous shaking (100 rpm) on a rotary shaker (DAIHAN Scientific, Korea) under 16-hours photoperiod in the growth chamber. Data were taken at different intervals and fixed five days until one month and were evaluted fresh and dry weight (g/flask) and the content of anthocyanin (µg/g DW) after each period.

2.6 Extraction of anthocyanin

The extraction of anthocyanin was carried out mother (intact) plant, callus and suspension culture from shake-flask as mentioned by Obouayeba and Bernard [33]. A known weight of the callus was taken and then dried until a constant weight was reached. Dried samples were grinded and 2ml of methanol were added with continuous stirring. The samples were then placed in the centrifuge at a rotation speed of 7500 rpm for 15 minutes. Chloroform was added to the supernatant to get rid of some compounds such as fat and chlorophyll. The samples were placed in a rotary evaporator, the supernatant was solved with a 1ml methanol and mixed by vortexing, the mixture was filtered with a 2.5µm filter and the supernatant was stored at 4ºC for use in subsequent analyses.

2.7 Analysis of total anthocyanin content (TAC)

TAC was determined by Hight Performance Liquid Chromatography (HPLC). The extraction of TAC was carried out from callus, culture suspension from shake-flask and leaves of intact plant by Dionex Ultimate 3000 equipped with an autosampler, quaternary pump and a diode array detector. Samples were injected into the HPLC under the following conditions, Column: nucludar 18-BD, (4.6×250 mm, particle size 5µm). Mobile phase: 0.01M phosphate buffer: Methanol 60:40 v/v. Flow rate 1.2 ml/min, and column temperature was maintained at 30 ºC. The detection wavelength was set at 250 nm. The extracts were analyzed by HPLC, according to previously reported methods [34].

2.8 Statistical analysis of data

All experiments were performed using the completely randomized design, with at least 10 replicates per treatment for callus culture experiment and three replicates for cell suspension culture experiments. Variance analysis of data was carried out using ANOVA program Duncan's multiple range test [35] as modified by Sendecor and Cochran [36]. Values marked with different letters were considered to be statistically different at $p \leq 0.05$

III. Results

3.1. Callus Induction and proliferation

The optimization of callus production by application of a suitable concentration of Plant Growth Regulators (PGRs) was considered. Leaves of *V. vinifera* led up to 100% of yellowish green, yellow pink spot and yellowish friable callus on all B5 media supplemented with 4.55, 9.1, and 18.2 µM 2,4-D, plus kin at 0.93 µM, respectively. However, a medium without PGRs (control) failed to produce any callus (Table 1 and Fig.1). The highest mean fresh weight (FW) of callus was 1.679 g/jar on B5medium supplemented with 9.1 µM 2,4-D and 0.93µM kin followed by B5 medium supplemented with 18.2 µM 2,4-D and 0.93 µM kin of 1.328 (g/jar), then B5 medium supplemented with 4.55 µM 2,4-D and 0. 93µM kin (0.788 g/jar). According to obtained results, It could be concluded that B5 medium supplemented with 9.1 µM 2,4-D and 0. 93 µM kin were the best concentration which resulted in the highest FW of callus comparing to the other treatments.

Table 1 Effect of 2,4-D at different concentrations and 0.93 µM kinetin on callus induction from leaf explants of *Vitis vinifera* after eight weeks of culture

PGR_S Concentration (µM)		Callus induction (%)	Fresh weight of callus (g/jar)	Colour and Texture
2,4-D	**Kin**			
0.00	**0.00**	0.00	0.00d	----------
4.55	**0.93**	100	0.788c	Yellowish green, friable
9.10	**0.93**	100	1.679a	Yellow pink spots, friable
18.2	**0.93**	100	1.328b	Yellowish friable

Means followed by the same letter within a column are insignficantly different at $P \leq 0.05$

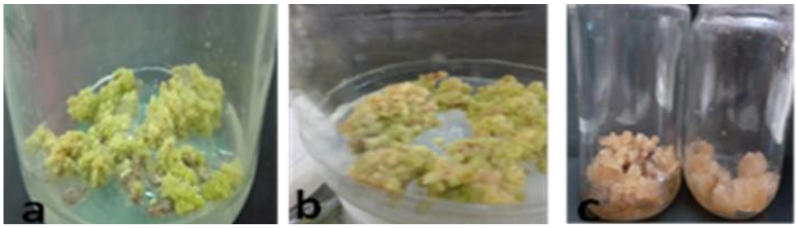

Fig. 1: Callus of *V. vinifera* on B5 medium supplemented with 0.93 µM kin plus
- **a.** 4.55 µM 2,4-D Yellowish green, friable
- **b.** 9.1 µM 2,4-D Yellow pink spot, friable
- **c.** 18.2 µM 2,4-D Yellowish, friable

Data in Table (2) clear that the highest percentage of increase in FW of callus (62.852%) was obtained on B5 medium supplemented with 9.1 µM 2,4-D and 0.93 µM kin and FW reaching 3.985 g/jar. Followed by B5 medium supplemented with 18.2 µM 2,4-D and 0.93 µM kin, which gave 1.931g/jar FW with percentage of increase in callus FW of 45.417%. From my observations, callus was pink when transfer to light (Fig. 2).

Table 2 Effect of different 2,4-D concentrations and 0.93 µM kintin callus proliferation of *Vitis vinifera* L. after six weeks of culture.

PRG_S concentration (µM)		Fresh weight of callus (g/ jar)	Increase in fresh weight of callus (%)
2,4-D	**Kin**		
0.0	**0.0**	0.00d	0.0 d
4.55	**0.93**	1.025b	30.076 c
9.1	**0.93**	3.985a	62.852 a
18.2	**0.93**	1.931b	45.417 b

Means followed by the same letter within a column are insignificantly different at $P \leq 0.05$

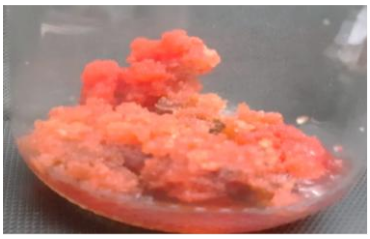

Fig. 2: Pink prolifration callus of *V. vinifera* on B5 medium supplemented with 9.1 µM 2,4-D + 0.93 µM kin

3.2 Elicitor and precursor feeding

Callus of *V. vinifera* was cultured on B5 medium supplemented with 2,4-D at 9.1 µM plus 0.93 µM kin, which gave the highest biomass of callus. For study the effect of (SA) and (L-Phe) have been added to the previous medium at different concentrations on FW and dry DW of callus and anthocyanin content. Data in Table 3 shows that callus FW , percentage of the increase in callus FW and DW reached the highest value when were cultured its on previous B5 medium supplemented with 302.7 µM L-Phe followed by 605.4 µM L-Phe and their values reached 9.538 g/jar, 90.76% and 1.423g and 7.938, 58.56%, and 0.883g, respectively. However, SA at different concentrations was produced the lowest values in all parameters but hight value compared with the control. To investigate the effect of addition SA and L-Phe on anthocyanin content in callus culture of *V. vinifera* after six weeks. Table 3 clear that all treatments of elicitors and precursor promoted biomass accumulation of anthocyanin and yield compared to control. Maximum accumulation of anthocyanin (8.108 µg/g dry weight) was recorded after callus treated with (L-Ph) at 302.7µM, which achieved amount five fold increase of anthocyanin production (2.64 µg/g dry weight and 1.64 fold) compared with the control and intact plant. Concerning the effect of SA on accumulation of anthocyanin, from the results in Table 3 observed that used 181 µM from SA gave hight anthocyanin content (5.658 µg/g dry weight) and appear 3.51 fold increase in production of anthocyanin compared tocontrol and the intact plant. Yield of anthocyanin in callus decrease by increasing the L-Phe and SA concentrations. An important note, the colour of callus was pink in spot and full pink when transfer it to light after four weeks from incubation in darkness (Fig. 3).

Table 3 Effect of elicitation and precursor feeding on fresh and dry weight of *Vitis vinifera* L. callus and anthocyanin accumulation on B5 medium supplemented with 9.1µM 2,4 -D and 0.93µM kin after six weeks of culture

Elicitor SA (µM)	Precursor L-Phe (µM)	Fresh weight of callus (g/jar)	Increase fresh weight of callus (%)	Dry weight of callus (g)	Anthocyanin content (µg/g dry weight)	* Yeild	Anthocyanin increase (fold)compared to intact plant (1.612 µg/g dry weight)
00.0	**00.0**	6.010e	20.2h	0.431d	2.640e	1.138	1.64e
181	**00.0**	6.793c	35.86e	0.694b	5.658c	3.927	3.51c
362	**00.0**	6.364d	27.28f	0.645c	3.514d	2.267	2.18d
724	**00.0**	6.265d	25.90g	0.489d	3.272d	1.600	2.03d
0.0	**302.7**	9.538a	90.76a	1.423a	8.108a	11.538	5.03a
0.0	**605.4**	7.938b	58.56b	0.883b	6.803b	5.371	4.22b
0.0	**908**	6.931c	38.62c	0.712b	5.690c	4.051	3.53c

Means followed by the same letter within a column are insignificantly different at $P \leq 0.05$

• Yeild = Dry weight of callus × Anthocyanin content

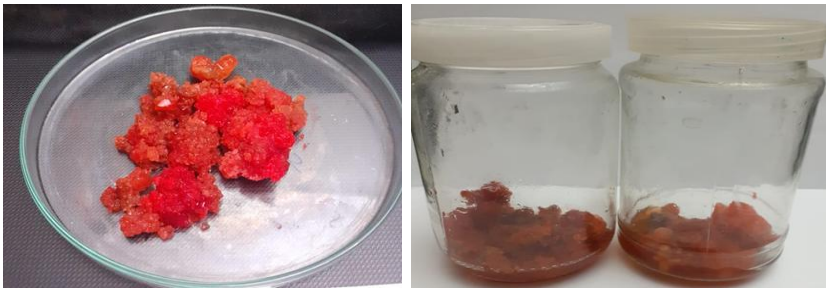

Fig. 3: Full dark pink of *V. vinifera* callus on B5 medium supplemented with 9.1 µM 2,4- D + 0.93 µM kin + 302.7 µM L-Phe

3.3 Cell suspension culture

The suspension cultures were established by transferring fresh friable pink in spot and full pink callus into liquid B5 medium supplemented with 9.1 µM 2,4-D plus 0.93 µM kin plus 302.7 µM L-Phe (the best medium composition from the previous experiment). Table 4 shows the effect of the duration and precursor of *V. vinifera* on biomass and anthocyanin accumulation in shake-flask suspension culture. Fifteen days of incubated on shake-flask induced maximum increase in FW and DW of 22.411and 3.984 g/flask, respectively. Also, it was observed that increase the incubation period by increasing the FW and DW up to 15 days and then decreased its by increasing the incubation period and recorded 31.037 fold for compared with control and intact plant (Fig. 4).

Table 4 Effect of different culture durations of *Vitis vinifera* suspension cultures grown in liquid B5 medium supplemented with 9.1 2,4-D and 0.93 µM kin in addition 302.7 µM L-Phe on biomass and anthocyanin accumulation

Culture duration (days)	Fresh weight of callus (g/flask)	Dry weight of callus (g)	Anthocyanin content (µg/g dry weight)	Anthocyanin increase (fold) compared to intact plant (1.612 µg/g dry weight)
0	9.538f	1.423e	8.108g	5.030g
5	13.791e	1.914e	15.412f	9.561f
10	16.457d	2.135d	34.144e	21.181e
15	22.411a	3.984a	50.032a	31.037a
20	20.153b	3.335b	43.867b	27.213b
25	19.711c	3.121b	40.168c	24.918c
30	19.403c	2.849c	37.477d	23.249d

Means followed by the same letter within a column are insignificantly different at $P \leq 0.05$

A minimum accumulation of anthocyanin in the shake-flask reached of five days incubation period, and it was 15.412µg/g DW and reached 9.561 µg/g DW fold compared with control and intact plant. In future, this study needs a lot of works and will throw more light on its participation in anthocyanin biosynthesis and may help in getting higher recovery of the final product.

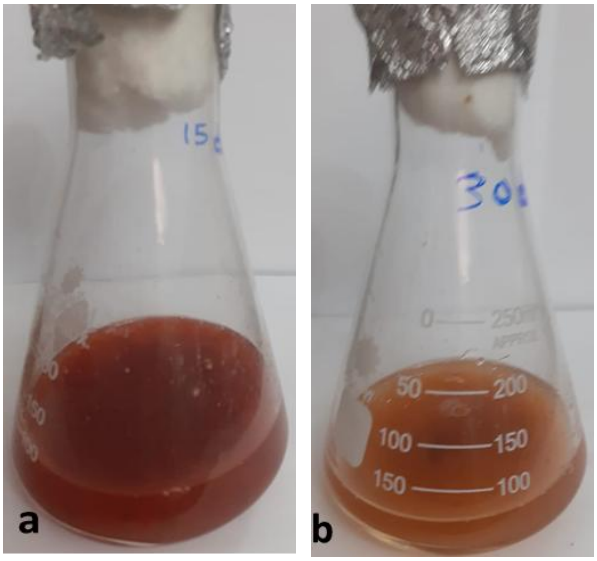

Fig. 4: Suspension culture of *V. vinifera* growen in B5 medium supplemented with 9.1 µM 2**,** 4-D + 0. 93 µM kin + 302.7 µM L-Phe

a. 15 days **b.** 30 days

IV. Discussion

The production of secondary metabolites by *in vitro* cultures usually occurs in a two-step process, biomass accumulation and secondary metabolites synthesis, in which both steps need to be optimized independently [18, 37]. Callus induction is necessary as the first step for the initiation of suspension cultures for the production of active constituents in tissue culture experiments [38]. From the previous results it is concluded that the concentration of 9.1 µM 2,4-D and 0.93 µM kin was the best conceration in achieving the highest weight callus similar results were obtained with [8] pointed out the callus induction and increase of callus weight could be referred to the influence of growth regulators on cell division and enlargement. Also, Rout et al. [39] found that the balance between auxin and cytokinin is an important factor for callus production. A combination of 2,4-D with kinetin promoted the cytoplasm of the vacuolated parenchyma cells of petiole cells in Arabidopsis thaliana become denser. [40]. The motivation behind why no callus induction in the medium without PGRs could be obvious the role of auxin and cytokinin in the stimulation of cell division which leads to the formation of callus [41]. Also, the effect of PGRs on increase callus production due to its role in the activation of some enzymes [42]. Concerning the an effective of 2,4-D Kurmi et al. [43] reported that induce callus of *V. vinifera* to need the presence of 2,4-D as auxin which plays an important role to induction and proliferation callus. Several reports indicated that auxin acts as a very important factor in callus induction, and cytokinins facilitate its effects [44.45]. The results of this study are consistent with the finding of [46] who found that using 2mg/l 2,4-D gave a positive result for fresh and dry weight of *V. vinifera*. The effect of the precursor with L-phe at different concentrations in suspension cultures of *V. vinifera* on callus a fresh weight and anthocyanin production was investigated. L-phe at low concentration has been gave the highest value of anthocyanin. This achievement is in line with El-Nabarawy et al. [47] who confirmed that higher concentrations of phenylalanine inhibited the fresh weight of *Zingiber officinale* callus in comparison with the fewer levels. In general, it could be clarify that L-Phe as amino acid speeds primer metabolism operation during phosphorenol pyruvic acid conversion to pyruvic acid; this acid act as a key compound in several biochemical pathways [48]. In addition, L-phe as aromatic amino acid possess a function as bioblock of protein and has an effect on hormone synthesis such as salicylate and auxin [49]. Cell suspension cultures is the most used culture system for elicitation treatment and secondary metabolites production [17]. It was observed that increasing the incubation period by increasing the FW and DW up to 15 days and then decreased its by increasing the incubation period. Similar results obtained by Saw et al. [28], how reported that the anthocyanin synthesis even decreases during the first 10 days but it is increased again between 15-18 days. Obinata et al. [27] found that production of procyanidin and anthocyanin was markedly increased when treated with SA as elicitor in cultured

grape. It has been explaining that SA plays an important role in the induction of systemic acquired resistance in plants and against abiotic stresses [50,51]. It is clear that SA is encourages the activities of one or more antioxidative enzymes [52]. SA plays a crucial role in the regulation of physiological and biochemical processes during the entire lifespan development of a plant [53]. Subsequently, it is possible that used the SA activated anthocyanin biosynthesis as a response to stress in callus cultures. From the data obtained, it was clarified that L-Phe is promoted the anthocyanin synthesis in cell suspension of *Vitis vinifera* plant. These results in agreement with Saw et al.[28], and Riedel et al. [50], who reported that phenylalanine as a precursor used to enhance the anthocyanin synthesis was 0.03 µg mg−1 higher than that of without phenylalanine grape *V. vinifera*. Wherefore, suspension cultures system is used for large-scale culturing of plant cells from which secondary metabolites can be produced and accumulated in large amounts. In addition, plant cell cultures are considered as a promising source for the sustainable production of secondary metabolites.

V. Conclusion

Through this work, I have established a reliable and effective *Vitis vinifera* cv. Cirmson callus and suspension cultures to produce valuable of anthocyanin by elicitation and precursor feeding. The results revealed that the use of L-phenylalanine (L-Phe) is an efficient strategy to improve anthocyanin production in cultures. Although, SA has potential effect on the synthesis of anthocyanin, but that effect is less than L-Phe. In this regard, B5 medium supplemented with 302.7 µM L-Phe induced the highest anthocyanin accumulation (8.108 µg/g dry weight) in callus cultures, representing a 5.029-fold increase as compared to the intact plant. The maximum accumulation of anthocyanin (50.032 µg/g dry weight) was observed after 15 days of culture in shake-flask suspension cultures with L-Phe at, 302.7 µM it reached 31.037-fold increase compared to the intact plant. The results obtained could serve as starting point for further studies regarding the widely production of the valuable compound anthocyanin from callus and suspension cultures of grape plant.

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Abbreviations: ANOVA: Analysis of variance; 2,4-D: 2,4-dichlorophenoxyacetic acid; kinetin: kin N6-furfuryladenine; SA: Salicylic acid; L-Phe: L-phenylalanine; FW: Fresh weight; DW: Dry weight; TAC: Total Anthocyanin Content; HPLC: Hight Preformance Liquid Coromatography; PGRs: Plant Growth Regulators**.**

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