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STIMULATION OF ANTHOCYANIN PRODUCTION IN STRAWBERRY CALLUS CULTURES

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ABSTRACT

A promising protocol for enhancement of anthocyanin production *in vitro* in different cultures of 'Selva', 'Oso grande' and 'Chandler' strawberry cultivars was established. The influence of different concentrations of 2,4-D and BAP addition to MS-medium (Murashige and Skoog, 1962) on callus production from shoot tip, leaf and root explants of different aseptic cultures of strawberry cultivars were investigated. The maximum value of growth dynamic of callus fresh weight (gm), dry weight (mg) and dry matter content (%) was recorded with Selva cultivar as compared with other strawberry cultivars. The optimum supplementation for callus production to MS-medium was 2.0 mg/1 2,4-D + 0.2 mg/1 BAP. Also, in this respect shoot tips of different strawberry cultivars gave better results of callus induction than other explants. Meanwhile, the optimum value of total anthocyanin accumulation percentage was recorded with leaf callus cultures of different strawberry cultivars. It was 157.98 µg/gm callus, 134.32 µg/gm callus and 98.73 µg/gm callus in Selva, Chandler and Oso grande, respectively.

Key words: In vitro, strawberry, callus cultures, anthocyanin,

INTRODUCTION

With recent advances in plant biotechnology, there have been few reports on the production of secondary metabolites such as alkaloids, quinones, terpenes, and flavonoids in plant tissue culture (Mori *et al,*1994). Anthocyanins have also been produced in various plants by tissue culture: wild carrot (Dougall and Weyrauch,1980, Kinnersley and Dougall , 1980), Vitis hybrida (Yamakawa *et al,* 1983 a, b),*Hibiscus sabariffa* (Mizukami *et al* 1988), *Vitis sp* (Tamura *et al*, 1989) and strawberry (Hong *et al*, 1989). There is an increasing use of natural anthocyanin based colorants in food products. The potent chemopreventive attributes of anthocyanins and related flavonoids have recently been demonstrated in both anticancer (Bomser *et al.,* 1996; Castonguay *et al,* 1997; Kamei *et al,* 1993; 1995 Kapadia *et al,* 1997; Koide *et al,* 1996 and cardioprotective (Bettini *et al,* 1991; Morazzoni and Bombardelli 1996; Morazzoni and Magistretti, 1990) bioassays, which have increased the interest in alternative means for their production and extraction .*In vitro* mass production of anthocyanin pigments from plant cell culture has been achieved (Shuler *et al*, 1990; Zhong and Yashida, 1995). Regulation of anthocyanin synthesis by manipulation of the chemical microenvironment, including carbohydrate, nitrogen source and level, is a routine approach to increase productivity *in vitro* (Do and Cormier, 1991; Mori and Sakurai, 1994; Mori *et al*,1993; Suzuki, 1995; Zhong and Yoshida, 1995). In particular, the suitable phytohormones source can exert a profound effect on both of callus production and anthocyanins

accumulation in different types of strawberry cultivars. The objective of our study was to clarify the relationship between anthocyanin production and calli derived from different explants shoot tip, leaf and root isolated from different strawberry cultivars selva(s), chandler and Oso grand and to examine the effects of 2,4– dichlorophenoxyacetic acid (2,4-D) and benzylamino purine (BAP) on callus growth and anthocyanin production .

MATERIALS AND METHODS

This investigation was carried out in Plant Cell and Tissue Culture Department , National Research Centre, Dokki, Egypt**.**

Plant material

Selva(s), Chandler and Oso grand strawberry cultivars were used in this investigation and micropropagated *In-vitro*.

The performance of planting material derived from tissue culture of strawberry have different stages as follow:-

1. Initiation stage: Cultures were initiated from small shoot tips consisting of meristem plus 2 or 3 leaf primordial which were dissected from stolons of greenhouse plants of strawberry. The tips were sterilized prior to dissection by immersion in 50% Clorox solution (commercial bleach) for 15 minutes followed by four washes in sterile distilled water. They were then cultured in 15x2.5 cm Test-tubes each with 10 ml of culture medium (Table-1) and closed with plastic caps . The shoot tips of all the varieties grew rapidly at the initial stage.
2. Rapid multiplication (shooting) stage: The aim of this stage is producing large proliferating shoot cultures, which were multiplied by sequential monthly subculture to fresh medium, were carried out in 300 ml jar each with 30 ml of culture medium (Table-1) and closed with aluminum foil. At this stage of culture, the shoots grew rapidly in all varieties.
3. Rooting stage: Obtained shoots from shoot tip (2-5 mm in length) were subsequently rooted by placing them (5 shoots/jar) in 30 ml of culture medium (Table-1) contained in 300 ml jar . After three weeks the small plantlets were obtained (Fig.1).

All cultures were maintained in a growth room at 25± 1°C with 16 hr. light daily (1000 Lux).

Table (1): Chemical composition of culture media (Boxus ,1974) of strawberry micropropagation.

Additions	Initiation stage	Shooting stage	Rooting stage
MS salt (g/L)	4.2	4.2	4.2
BAP (mg/L)	0.1	1.0	0.0
IBA (mg/L)	1.0	1.0	1.0
Sucrose (g/L)	30.0	30.0	30.0
Agar (g/L)	7.0	7.0	7.0
pH	5.6-5.8	5.6-5.8	5.6-5.8

MS= Murashige and Skoog (1962). BAP= 6-Benzylaminopurine .IBA= Indol butyric acid .
***The pH of the medium was adjusted before autoclaving .**
***All media sterilized by autoclaving at 121 °C /20 min.**

Fig.(1): Strawberry plantlets initiated *in vitro*

Callus production

Shoot tip, leaf and root explants were excised from sterilized strawberry plantlets resulted from tissue culture. Three explants from each type were transferred into 200 ml jar containing 25ml of MS- medium (with 4 % sucrose and 0.7% agar) supplemented with different concentrations of 2,4–D at 0.5, 1.0 and 2.0 mg/l in combination with 0.2 and 0.5 mg/l BAP. The pH of the medium was adjusted to 5.8 with 0.1 N KOH or 0.1 N NaOH prior autoclaving at 121°C for 20 min.. All cultures were incubated at room temperature (25 ±1 °C) under 16 hr light and 8 hr dark cycle with light intensity 1000 Lux. Subcultures were done every month.

Determination of callus growth

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

1. Fresh weight (gm).

2. Dry weight (mg).
3. Dry matter content (%).

Statistical analysis

This experiment was designed in completely randomized and obtained data were statistically analyzed using standard error ± SE (standard error) according to the method described by Snedecor and Cochran (1980).

Determination of anthocyanin (µg/gm) callus fresh weight.

Half gram of strawberry callus 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 the sample, vortexing then centrifuged at 1000 rpm for 10 min. Absorbance 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 method which was described by (Mori *et al*, 1993) . The major anthocyanin had been previously identified. Total anthocyanin yield was expressed as (µg/gm) callus fresh weight.

RESULTS

Callus production

1– Callus fresh weight (gm):

To determine callus fresh weight, different explants, such as shoot tip, leaf and root were cultured on MS-medium containing different concentrations from 2, 4-D at 0.5, 1.0 and 2.0 mg/l in combination with BAP at 0.2 and 0.5 mg/l. Data tabulated in Table (2) show that maximum values of callus production as fresh weight (gm) were observed in selva (s) as a compared with Chandler or Oso grand cultivars. Shoot tip of different cultivars showed better results of callus production than leaf or root explants.

Table (2): Effect of different concentrations of 2,4-D and BAP on callus fresh weight (gm) of different strawberry cultivars.

Cultivars	Medium (mg/l)		Fresh weight (mg)		
			Shoot tip	Leaf	Root
Selva (s)	0.5	0.2	1.17 ± 0.04	1.06 ± 0.038	0.40 ± 0.017
	0.5	0.5	1.36 ± 0.05	1.19 ± 0.026	0.503 ± 0.04
	1.0	0.2	1.77 ± 0.04	1.46 ± 0.040	0.72 ± 0.026
	1.0	0.5	1.85 ± 0.05	1.66 ± 0.044	0.92 ± 0.020
	2.0	0.2	2.94 ± 0.25	2.17 ± 0.115	1.12 ± 0.07
	2.0	0.5	2.14 ± 0.09	1.63 ± 0.113	1.08 ± 0.07
Chandler	0.5	0.2	1.07 ± 0.04	0.84 ± 0.06	0.32 ± 0.017
	0.5	0.5	1.25 ± 0.04	1.12 ± 0.02	0.38 ± 0.03
	1.0	0.2	1.58 ± 0.033	1.37 ± 0.03	0.56 ± 0.03
	1.0	0.5	1.78 ± 0.05	1.461 ± 0.06	0.74 ± 0.05
	2.0	0.2	2.25 ± 0.05	1.93 ± 0.04	1.25 ± 0.04
	2.0	0.5	2.00 ± 0.09	1.55 ± 0.04	1.09 ± 0.05
Oso grand	0.5	0.2	0.90 ± 0.11	0.85 ± 0.01	0.22 ± 0.03
	0.5	0.5	1.21 ± 0.02	0.96 ± 0.07	0.30 ± 0.03

	1.0	0.2	1.26 ± 0.05	1.2 ± 0.04	0.44 ± 0.05
	1.0	0.5	1.44 ± 0.06	1.27 ± 0.06	0.64 ± 0.06
	2.0	0.2	1.9 ± 0.11	1.81 ± 0.04	1.13 ± 0.02
	2.0	0.5	1.64 ± 0.06	1.45 ± 0.04	0.84 ± 0.05

± Standard error **Each value is the average of 5 replicates**

The optimum supplementation of 2,4–D as an auxin in combination with BAP as a cytokinin was 2.0 and 0.2 mg/l, respectively .

The descending order of callus fresh weight (gm) was 2.94, 2.17 and 1.12 in Selva (s) cultivar, and it was 2.25, 1.93 and 1.25 in Chandler cultivar. Meanwhile the minimum value of callus fresh weight (gm) was recorded in Oso grand it was 1.90, 1.81, 1.13 in shoot tip, leaf and root callus cultures, respectively (Fig.2).

Fig. (2): Strawberry callus cultures induced from shoot tip, leaf and root explants

2 – Callus dry weight

Dry weight was measured as indicator for true growth pattern of callus production. Table (3) shows that, the optimum concentration of 2,4–D was 2.0 mg/l and BAP was 0.2 mg/l. The best results of dry weight were observed in Selva (s), Chandler and Oso grand, respectively. The best explants showed the maximum value of dry weight were recorded with shoot tip, leaf then root cultures, respectively. The recorded values were 27.06, 20.01 and 5.9 (mg) in shoot tip, leaf and root callus of Selva cultivar. And it was 20.04, 17.3 and 9.1 (mg) in shoot tip,leaf and root callus cultures of Chandler. Meanwhile the minimum values of dry weight (mg)were recorded with shoot tip, leaf and root culture of Oso grand which were 16.5 , 16.3 and 7.04 (mg) , respectively.

3- Callus dry matter content (%):

Optimum values of dry matter content (%) were recorded with Selva (s), Chandler and Oso grand, respectively. As shown in Table (4) the best results of Selva dry matter content were 9.49 %, 9.72 % and 9.2 % in leaf,

shoot tip and root callus when the shoot tip and leaf were cultured on MS medium supplemented with 1.0 mg/l 2,4,-D + 0.5 mg/l BAP in case of root culture it was 9.2 % with MS medium + 1 mg/l 2, 4 – D+ 0.2 mg/l BAP. On the other hand, MS medium supplemented with 2.0 mg/l 2, 4 -D + 0.2 mg/l BAP showed the best results in shoot tip and leaf callus cultures of Chandler cultivar, it was 9.13 % and 9.26 %, respectively. But in case of root callus cultures MS-medium supplemented with 1 mg/l 2,4-D + 0.2 mg/l BAP gave the best results of dry matter content (9.04 %). Meanwhile, the minimum value of dry matter content (%) was recorded with Oso grand it was 8.92 in leaf callus when cultured on MS-medium supplemented with 0.5 mg/l 2,4-D + 0.5 mg/l BAP.

Table (3): Effect of different concentrations of 2,4-D and BAP on callus dry weight (mg) of different strawberry cultivars.

Cultivar	Medium (mg/l)		Dry weight (mg)		
			Shoot tip	Leaf	Root
Selva (s)	0.5	0.2	0.0896 ± .0022	0.0715 ± 0.0008	0.037 ± 0.0008
	0.5	0.5	0.115 ± 0.0045	0.1001 ± 0.0032	0.0387 ± 0.0003
	1.0	0.2	0.1604 ± .0012	0.1304 ± 0.0011	0.0661 ± 0.0005
	1.0	0.5	0.176 ± 0.0067	0.1614 ± 0.0009	0.0676 ± 0.0006
	2.0	0.2	0.2706 ± 0.02	0.2001 ± 0.008	0.059 ± 0.0042
	2.0	0.5	0.202 ± 0.009	0.152 ± 0.011	0.873 ± 0.003
Chandler	0.5	0.2	0.078 ± 0.0032	0.065 ± 0.004	0.022 ± 0.003
	0.5	0.5	0.0895 ± 0.002	0.099 ± 0.0021	0.024 ± 0.003
	1.0	0.2	0.139 ± 0.0073	0.122 ± 0.002	0.05 ± 0.0035
	1.0	0.5	0.157 ± 0.0038	0.131 ± 0.009	0.061 ± 0.0038
	2.0	0.2	0.204 ± 0.0086	0.173 ± 0.0066	0.091 ± 0.005
	2.0	0.5	0.182 ± 0.0086	0.144 ± 0.005	0.084 ± 0.0052
Oso grand	0.5	0.2	0.064 ± 0.0058	0.058 ± 0.004	0.016 ± 0.0018
	0.5	0.5	0.083 ± 034	0.085 ± 0.0039	0.017 ± 0.0029
	1.0	0.2	0.108 ± 0.0087	0.099 ± 0.003	0.042 ± 0.0038
	1.0	0.5	0.136 ± 0.0058	0.126 ± 0.0046	0.049 ± 0.0034
	2.0	0.2	0.165 ± 0.005	0.163 ± 0.006	0.074 ± 0.0058
	2.0	0.5	0.156 ± 0.0048	0.135 ± 0.004	0.069 ± 0.0035

± Standard error Each value is the average of 5 replicates

Table (4): Effect of different concentrations of 2,4-D and BAP on callus dry matter content (%) of different strawberry cultivars.

Cultivars	Medium (mg/l)		Dry matter content (%)		
			Shoot tip	Leaf	Root
Selva (s)	0.5	0.2	7.68 ± 0.09	6.76 ± 0.16	9.19 ± 0.19
	0.5	0.5	8.44 ± 0.11	8.41 ± 0.11	7.78 ± 0.59
	1.0	0.2	9.14 ± 0.14	8.92 ± 0.19	9.2 ±0.28
	1.0	0.5	9.49 ± 0.21	9.72 ± 0.19	7.38 ± 0.10
	2.0	0.2	9.23 ± 0.19	9.28 ± 0.13	8.5 ± 0.20
	2.0	0.5	9.46 ± 0.05	9.34 ± 0.27	8.12 ± 0.26
Chandler	0.5	0.2	7.29 ± 0.05	7.81 ± 0.088	6.71 ± 0.54
	0.5	0.5	7.17 ± 0.09	8.81 ± 0.008	6.30 ± 0.36
	1.0	0.2	8.77 ± 0.22	8.87 ± 0.29	9.04 ± 0.28
	1.0	0.5	8.81 ± 0.19	8.96 ± 0.29	8.25 ± 0.18
	2.0	0.2	9.06 ± 0.23	8.96 ± 0.11	7.26 ± 0.18
	2.0	0.5	9.13 ± 0.03	9.26 ± 0.08	7.73 ± 0.11
Oso grand	0.5	0.2	7.09 ± 0.23	6.85 ± 0.44	7.13 ± 0.05

	0.5	0.5	6.88 ± 0.27	8.92 ± 0.25	5.43 ± 0.43
	1.0	0.2	6.81 ± 0.40	7.25 ± 0.23	7.44 ± 0.42
	1.0	0.5	7.62 ± 0.23	8.59 ± 0.10	6.61 ± 0.1
	2.0	0.2	7.25 ± 0.15	8.41 ± 0.15	5.94 ± 0.27
	2.0	0.5	7.84 ± 0.1	8.71 ± 0.09	6.33 ± 0.03

± Standard error **Each value is the average of 5 replicates**

Also, in the same respect it was 7.84 % in shoot tip when cultured on MS-medium supplemented with 2.0 mg/l 2, 4-D + 0.5 mg/l BAP. But it was 7.44 % in case of root cultures which were cultured on MS-medium supplemented with 1.0 mg/l 2, 4-D + 0.2 mg/l BAP.

Anthocyanin determination

The effect of different concentrations 0.5, 1.0 and 2.0 mg/l from 2, 4-D as an auxin in combination with 0.2 or 0.5 mg/l BAP as a cytokinin on total anthocyanin content (µg/gm callus) was investigated.

Data in Fig (3) show that the effect of supplementation of MS-medium with 2.0 mg/l 2, 4-D + 0.2 mg/l BAP gave the best results as for total anthocyanin induction and accumulation in different strawberry cultivars.

Fig (3): Influence of different concentration of 2,4D and BAP on anthocyanin production (μg/g) of shoot tip, leaf and root callus cultures of various strawberry cultivars.

The optimum values of anthocyanin synthesis were recorded in Selva (s), Chandler and Oso grand, respectively. Leaf callus cultures gave more anthocyanin accumulation than other tissues i.e. shoot tip and root callus culture.

DISCUSSION

The influence of different concentrations of 2, 4 –D as an auxin in complementation with BAP as a cytokinin on callus induction and anthocyanin production from leaf explants of *Fragaria ananassa cu.* Skikinari strawberry cultivar was investigated by Mori *et al,* (1994), they reported that the best results were obtained from the addition of 1 mg/l 2,4–D + 1 mg/l BAP to MS-medium. The differences in our results and those obtained by Mori *et al,* 1994 may be due to the variation of plants and cultivars. On the other hand, they investigated the different concentrations of 2, 4 –D and BAP on anthocyanin accumulation on above mentioned cultivar, they reported that the optimum concentration of 2, 4–D was 1 mg/l and BAP ranged from 0.0 to 0.1 mg/l. This result was in close agreement with our result since light concentration of 2, 4 –D (2.0 mg/l) combined with low concentrated (0.2 mg/l) BAP stimulated anthocyanin production in different studies strawberry cultivars.

In this respect, 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*, these results are in agreement with our results, the balanced 2, 4 –D at the rate of 2.0mg/l was the optimum supplementation to MS medium for both of callus growth and anthocyanin production. On contrast of above mentioned results, Meyer and Van Staden (1995) reported that BAP stimulated anthocyanin production, but depressed cell growth in callus cultures of *Oxalis lineasis*.

CONCLUSION

We can conclude that using leaf explants of Selva (s) cultivar is more suitable explants and cultivars than both of other explants and other cultivars. In this respect, the conditioning of MS-medium by 2.0mg/l 2, 4–D in complementation with 0.2 mg/l BAP modified MS-medium towards the best for both callus induction and anthocyanin production.

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تنشيط انتاج الأنثوسيانين فمزارع كالس الفراوله

عادل الصاوى و حسين سيد طه

قسم زراعه الخلأيا و الأنسجه النباتيه, المركز القومى للبحوث-الدقى-القاهره-مصر.

تم تاثيث طريقه واعده لانتاج الأنثوسيانين معمليا فى بعض اصناف المزارع المختلفه للفراوله 'Selva', 'Oso grande' and 'Chandler'.تم دراسه تاثيراضافه التركيزات المختلفه من 2,4-D , BAP الى بيئه موراشيج-سكوج المغذيه فى انتاج مزارع الكالس من القمم المرستيميه,الاوراق و الجذور . كانت

اعلى قيمه لديناميكيه الوزن الطازج (جرام) لمزارع الكالوس والوزن الجاف (ملليجرام) وكذلك النسبة المئوية للرطوبة في صنف Selva مقارنة بالأصناف الأخري.

كانت أنسب اضافة لبيئة موراشيج وسكوج المغذية هي 2 ملليجرام /لتر من 2,4-D + 2, ملليجرام / لتر BAP.

وقد سجلت القمة المرستيمية في نفس الاتجاه أحسن النتائج لأنتاج الكالوس بالمقارنة بالأجزاء النباتية الأخري، بينما سجلت مزارع الأوراق أحسن نتائج في انتاج الأنثوسيانين والتي كانت 157,98 ، 134,32 ، 98,73 ميكروجرام / جرام كالوس في أصناف 'Oso grande' ,'Selva'و'Chandler' على الترتيب.