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EFFECTS OF EXOGENOUS GROWTH REGULATORS ON CELL SUSPENSION CULTURE OF "*YIN-HONG*" GRAPE (*VITIS VINIFERA* L.) AND ESTABLISHMENT OF THE OPTIMUM MEDIUM

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Abstract

Callus induced by stem of *"Yin-hong"* grape (*Vitis vinifera* L.) was used as materials and B5 medium as basic medium. The major growth parameters of cell suspension cultures with various levels of 1-Naphthaleneacetic acid(NAA) and 6-Benzyl aminopurine(6-BA)were investigated to provide a basis for the optimum medium of suspension cell cultures of *"Yin-hong"* grape regarding cell number, packed cell volume (PCV), dry cell weight (DCW), cell viability, and morphology. All data were analysed by of two-way analysis of variance (ANOVA). Results showed that the treatment of 6-BA and NAA would effect the cell growth dynamics, probably causing logarithmic phase in advance at higher levels of 6-BA. Different concentration of 6-BA and NAA had significant effects on cells' number, PCV, DCW and viability ($p<0.05$), while no-significant effect was observed on the cells morphology. The optimum medium for suspension cell cultures of "*Yin-hong*" grape was identified asB5+1.5 mg/L6-BA+1.5 mg/LNAA+ 250 mg/L casein hydrolysate + 30 g/L sucrose. With the optimum medium, the maximum number of suspension cells after the logarithmic growth phase was 34.78×10^8 / mL, the highest cell viability reached 86.45%.; DCW reached 3.84 g/L and PCV reached 0.092 mL/mL after eight days cultivating.

Key words: *"Yin-hong"* grape, Cell suspension, Exogenous growth regulator, Optimum medium.

Introduction

Plants are important sources of many essential compounds. Novel technologies have been proposed to obtain these compounds, usually secondary metabolites, in large quantities have by using *In vitro* tissue culture. Considering various forms of *In vitro* plant tissue cultures, cell suspension culture is most amenable to large-scale production of natural compounds, primarily due to its superior culture homogeneity (Kharenko *et al*., 2011). There are a number of plant cell cultures producing a higher amount of secondary metabolites than in intact plants (Rao& Ravishankar, 2002). However, unsolved problem still exists in the production of metabolites by cell cultures resulting from the instability of cell lines, low yields, slow growth and scale-up problems (Ravishankar & Venkataraman, 1993). The synthesis of secondary metabolites is strongly related to the cell physiological state, which is affected by the culture medium (Satdive *et al*., 2007; Zha *et al*., 2007), exogenous phytohormones (Pasqua *et al*., 2005), precursor feeding and elicitation (Baldi & Dixit, 2008; Karwasara *et al*., 2010).

While much work has been done on the nature and the concentration of endogenous plant growth regulators in differentiated plant tissues (Weiler, 1981; Garshasbi *et al*., 2012), only few studies on these compounds in plant cell cultures have been carried out. In addition, in published research about exogenous phytohormones treatment, the available information on cellular growth parameters of suspension cultures is still limited.

"*Yin-hong*" was a new grape variety registered and released for commercial production in Zhejiang province, China in 2011. This grape was developed from *Vitis vinifera*, bred by author's university. Preliminary study showed that its plants and fruits contain plenty of anthocyanins. In this research work, callus induced by stem of "*Yin-hong*" grapes was used as materials, the effect of various cell's growth parameters of suspension cultures with various levels of growth regulators were investigated. A stable suspension cell line of "*Yin-hong*" grape was obtained for further research of anthocyanin biosynthesis and application.

Materials and Methods

Plant materials and cell suspension culture: Callus induced by stem of "*Yin-hong*" grapes had beening subcultured every 4 week in our laboratory for half years. Friable pieces of callus 1.6 g were inoculated in 100mL erlenmeyer flasks containing 40 mL B5 medium, supplemented with 30g/L sucrose, 250mg/L casein hydrolysate (Qu *et al*., 2011; Saw *et al*., 2012).The treatment of two-factors and five-levels of growth regulators was added to suspension cell culture medium (Table 1). The pH of the medium was adjusted to 5.8±0.1 before autoclaving (121°C, 1.2 kg cm−2 pressure for 20 min). Cultures were incubated in dark on a gyratory shaker at 120 rpm at 25±1°C (Zhao *et al*., 2010). Each growth regulators treatment was replicated in three flasks.

Table 1.The treatment of two-factors and five-levels of growth regulators added to suspension cell culture medium.

NAA (mg/L)	6-BA(mg/L)				
	0.0	**0.5**	**1.0**	**1.5**	**2.0**
0.0	1	6	11	16	21
0.5	2	7	12	17	22
1.0	3	8	13	18	23
1.5	4	9	14	19	24
2.0	5	10	15	20	25

Assay of the cell couting: During the period of treatment of various level of growth regulators, the cells suspensions were sampled three times every 24 hours from first day to eight day, and the number of cells were counted using counts chamber. Before cell counting, cells were dissociated by cellulase and pectolyase. Filled the counting cell chamber with the mixture, position carefully the cover glass on top of the chamber, to avoid the formation of bubbles. Observed under the microscope with the×10 objective to locate the squared field. Counted all the cells contained in 10 squares. Added the values of the 10 squares (do not obtain the average). This number represented the number of cells in 10 µL, so multiply by 100 to determined the cell number per milliliter (Víctor *et al*., 2006).

Assay of the cell biomass: After 8 days of incubation, Cellin the volume of 10 ml cell suspensions were harvested by vacuum filtration through filter paper, washed with distilled water, put into the 80^{o}C -oven overnight to gain the dry cell weight (DCW) (Cai *et al*., 2011). Packed cell volume (PCV), the cell volume (mL) in per ml of cell cultures, was determined by centrifuging 10 mL of the culture in a 15-mL graduated conical centrifuge tube at 2000g for 5 min (Víctor *et al*., 2006).

Assay of cells viability: Cell suspensions in the logarithmic phase (about3-4 days after inoculation) were sampled to detect cell viability. A concentrated stock solution of Fluorescein diacetate(FDA) (500 g L^{-1}) was prepared in dimethyl sulfoxide (DMSO). For the staining protocol, cell suspensions (1ml) were incubated with 10 µL of FDA stock solution in the dark for 10 min at room temperature (Fontes *et al*., 2010). Cell were observed and photographed under a Nikon 80iepifluorescence microscope with appropriate filter settings. The intact plasma membrane was permeable to FDA, and FDA was converted to a green fluorescent dye, fluorescein, by cytoplasmic esterases, displaying a green fluorescence in viable cells.

$$\text{The rate of viable cells} = \frac{\text{The number of viable cells}}{\text{Total number of cells}} \times 100\%$$

Statistical analysis: All analyses were performed in triplicate, and data were reported as the mean ± standard deviation (SD).All data were subjected to analysis by two-way analysis of variance (ANOVA) of SPSS Version17.0 (SPSS Inc., Chicago, IL, USA), using Origin Pro 8 software for Windows (Origin Lab Corporation, MA, USA).

Results and Analysis

Effects of different treatments of NAA and 6-BAon cell growth: The number of cells in all samples treated with 25 combination of growth regulator were counted during eight days' cultivating. All the growth dynamics of suspension cell were described by a sigmoid curve, which showed three phase: lag phase (1-3 or 4 d), logarithmic growth phase (3-5 d or 4-6 d) andstationary phase (5 or 6-8 d) (Fig. 1). After 7 days of cultivating, the cell number remained stable. So the advisable subculture time was 7-8 d. The growth curve profile of "*Yin-hong*" grape was similar to that of most other plant cell cultures, whileits logarithmic growth phase appeared comparatively shorter than other species (Kanabus *et al*., 1986; Botha & O'Kennedy, 1998; Rontein *et al*., 2002). Thus, suspended cultures of grape's cell were an effective approach to provide large amounts of cells in less time, which was conducive to large-scale production of secondary metabolites.

In this experiment, grape's logarithmic phase was partly affected by treatment of growth regulator. Cells in the treatment 11-25 were mostly in logarithmic growth on 3th day, but on 4thday in other treatments. It was likely that the higher levels of 6-BA (1.5-2.0 mg/L) promotes cell logarithmic phase in advance. In the logarithmic phases, the high rates of cell division resulted in the growth of suspension culture (Szabados *et al*., 1993). Logarithmic growth of cell was associated with higher levels of exogenous6-BA, which indicated that the presence of the growth regulator outside the cellwas more important than its level with in the cell at certain stages in the growth process (Weiler *et al*., 1981).

Effects of different treatments of NAA and 6-BA on maximum number and viability of cells: Stained with FDA, viable cells displayed intensive fluorescence observed under UV light (Fig. 4B).After logarithmic growth phase, the maximum number and viability of suspension cells showed that there were significant fluctuation among various treatment of growth regulator (Fig. 2). At the level of 1.5 mg/LNAA, the maximum number and cell viability were both highest in treated cells. A decreasing tendency for values was observed in the cells treated with NAA concentration higher than 1.5 mg/L. In treatment 19, the maximum number and cell viability were both highest (34.78×10^8 / mL; 86.45%), and in treatment 22 (34.73×10^8 / mL; 85.34%) was the second highest.

The analysis of the variance and significance test showed that the effects of different concentrations of 6-BA and NAA as well as their interaction on the maximum number and cell viability reached extremely significant levels ($p<0.01$) (Tables 2, 3). The maximum number and cell viability at the level of 1.5mg/L NAA were highest and had a significant differences with other three levels ($p<0.05$), while no-significant difference was observed among other three levels ($p>0.05$).

Effects of different treatments of NAA and 6-BA on cell biomass: After 8 days of incubation, DCW of treatment 22 (3.84 g/L) and treatment 19 (3.81 g/L) were the highest two. The PCV of treatment 19 were the highest (0.092 mL/mL), followed by treatment 14 and 22 whose PCV were both 0.087 mL/mL (Fig. 3). This observation implied a positive correlation between PCV and DCW. Both DCW and PCV displayed initial ascending and then descending tendency as NAA concentration increased, which was similar with the effect of treatments on maximum number of cells. At the level of 1.5-2.0mg/L 6-BA, biomass of suspension cell reached peak.

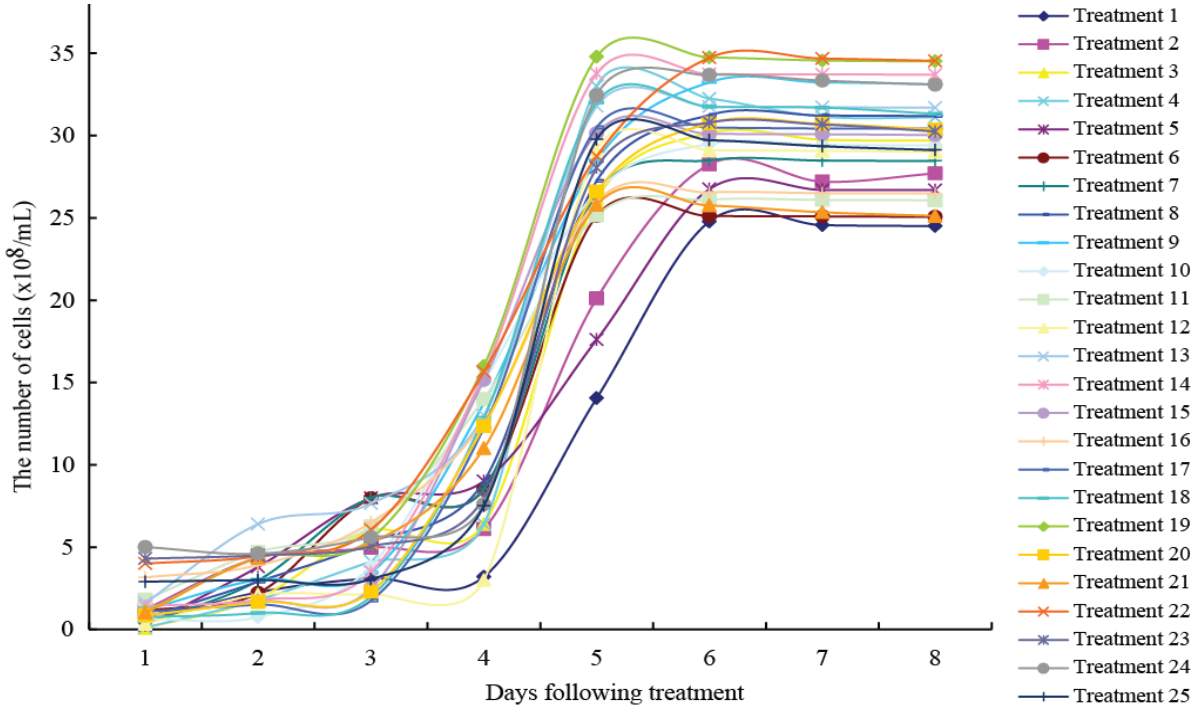

Fig. 1. Growth dynamic of "*Yin-hong*" grape's cell treated by 25 combination of growth regulator during 8 days' cultivating.

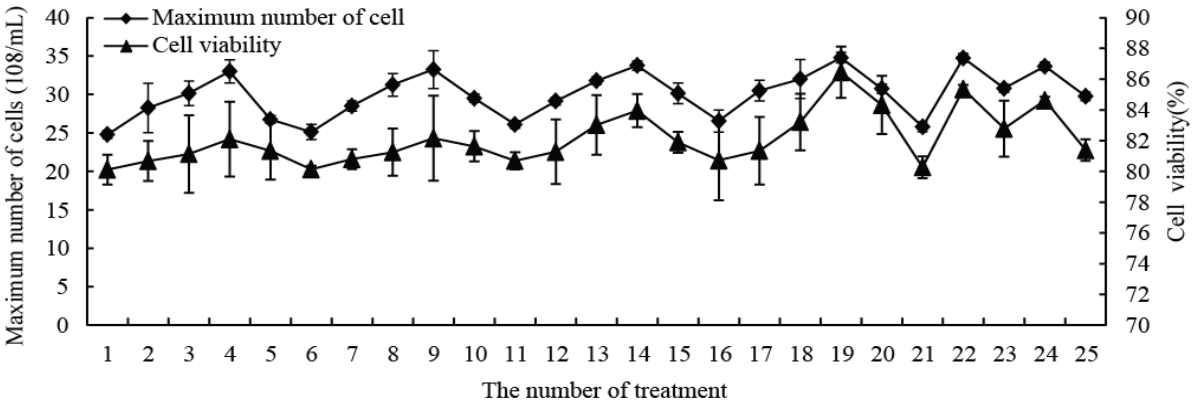

Fig. 2. Effects of different treatments on maximum number and viability of suspension cells after logarithmic growth phase. The data shown are means of three replicates, and ± SD values are presented as error bars.

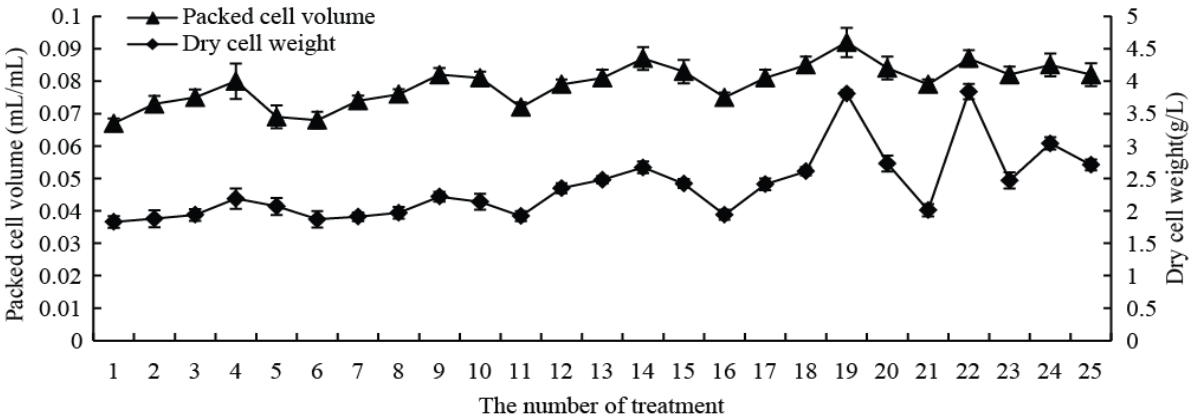

Fig. 3. Effects of different treatments on biomass of suspension cells. The data shown are means of three replicates, and ± SD values are presented as error bars.

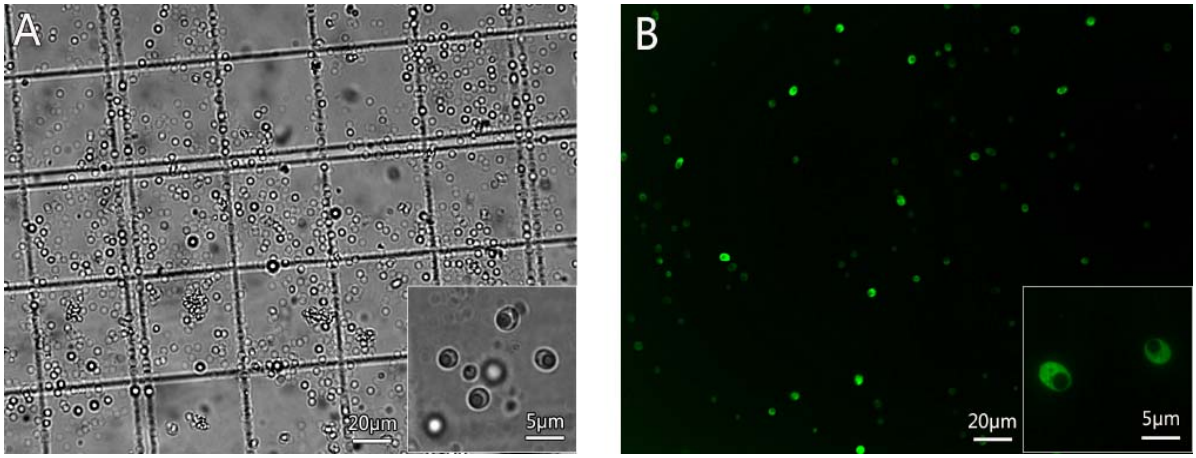

Fig. 4. Suspension cell of "Yinhong" grape observed under the light microscope in a blood counts chamber for counting (**A**).*Inset*: a close-up view of an intact suspension cell showing integrity of the vacuolar apparatus. Suspension cell of "Yinhong" grape observed under UV light (epifluorescence) after staining with fluoresce in diacetate (FDA) to measure viability (**B**). *Inset*: a close-up view of an intact suspension cell highlighting the integrity of both the plasma membrane and tonoplast.

Table 2. Duncan's post hoc test on maximum number of cells at different levels of 6-BA and NAA (α = 0.05).

Levels (mg/L)	Maximum number of cells	
	6-BA	NAA
0.0	28.5860 ± 3.28515a	25.6680 ± 1.00090a
0.5	29.5220 ± 3.06519ab	30.2220 ± 2.82422d
1.0	30.1800 ± 2.74269bc	31.1940 ± 1.46555b
1.5	30.9160 ± 3.02859c	33.6900 ± 1.32530c
2.0	30.9480 ± 3.29949c	29.3780 ± 1.57484d

The values are mean of three replicates± standard deviation
Different letters in the column indicate significant differences($p<0.05$)

Table 3. Duncan's post hoc test on the cell viability at different levels of 6-BA and NAA (α = 0.05).

Levels (mg/L)	Cell viability (%)	
	6-BA	NAA
0.0	81.0713 ± 1.76050a	80.3887 ± 1.13903a
0.5	81.1913 ± 1.46667a	81.8833 ± 2.21672b
1.0	82.1647 ± 1.71455ab	82.2760 ± 1.90071b
1.5	83.2080 ± 2.76423b	83.8533 ± 2.31758c
2.0	82.8807 ± 2.12565b	82.1147 ± 1.62182b

The values are mean of three replicates± standard deviation
Different letters in the column indicate significant differences ($p<0.05$)

Table 4. Duncan's post hoc test on the DCW at different levels of 6-BA and NAA (α = 0.05).

Levels (mg/L)	DCW (g/L)	
	6-BA	NAA
0.0	1.9807 ± 0.17027a	1.9133 ± 0.10125a
0.5	2.0240 ± 0.16238a	2.4773 ± 0.74514b
1.0	2.3673 ± 0.26601b	2.2960 ± 0.30227c
1.5	2.7013 ± 0.64370c	2.7867 ± 0.62834d
2.0	2.8133 ± 0.64143d	2.4133 ± 0.29911b

The values are mean of three replicates± standard deviation
Different letters in the column indicate significant differences ($p<0.05$)

Table 5. Duncan's post hoc test on the PCV at different levels of 6-BA and NAA (α=0.05).

Levels (mg/L)	PCV (mL/mL)	
	6-BA	NAA
0.0	0.07300 ± 0.005644a	0.07240 ± 0.004968a
0.5	0.07627 ± 0.005587b	0.07887 ± 0.005357b
1.0	0.08053 ± 0.005630c	0.07987 ± 0.004138b
1.5	0.08347 ± 0.006255d	0.08533 ± 0.005525c
2.0	0.08280 ± 0.003570d	0.07960 ± 0.006379b

The values are mean of three replicates± standard deviation
Different letters in the column indicate significant differences($p<0.05$)

As shown in Table 4, the analysis of variance indicated that there were significant differences in the effects of different concentrations of 6-BA and NAA as well as their interaction on the DCW and PCV ($p<0.01$).The DCW at the levels of 0.0-0.5mg/L 6-BA was significantly lower than that of 1.0-2.0 mg/L ($p<0.05$); The DCW at the level of 1.5 mg/L NAA was largest, ahead of that of 0.5 mg/L. The PCV at the level of 1.5 mg/L 6-BA was the highest which had a significant difference with other levels ($p<0.05$); The PCV at the levels of 1.5 mg/L NAA was the highest with significant difference with other levels ($p<0.05$) (Table 5). These results suggest that suspension medium supplied 1.5 mg/L 6-BA, 1.5 mg/L NAA(treatment19) was suitable for higher biomass.

The results indicated that the type and level of growth regulator were the two important factors affecting grape's cell biomass or secondary metabolite yields.

Observation of cell morphology after different treatments of NAA and 6-BA: In each phase of the suspension cell growth, changes in cell diameter were recorded using ocular micrometer. In the lag phase, all of cell diameter were practically same (about 0.8mm); in the logarithmic phase, difference among individual cells diameter became noticeable, varying from 0.8mmto 1.0 mm; in the stationary phase, cell diameter recovered to be same(about 1.0mm). The cell size changed a little in the entire cell growth cycle, and no obvious distinction of cell size was observed in suspension cell treated by various combination of growth regulator.

Observation of suspension cells' morphology under optical microscopy was shown in Fig. 4A: suspension cells arranged hierarchical, presented individually or in clusters (especially in logarithmic phase). Over incubation time, cells showed spherical shape gradually. In lag phase, the cells were at a standstill. In logarithmic phase, lots of cell showed a rapid, non-directional, reciprocating moving at the same time. In stationary phase, the speed of movement gradually slowed down. These results are in corroboration with the earlier results (Deroles, 2009), who reported: liquid suspension cultures usually consist of small multicellular aggregates rather than a true single cell suspension. The size of these aggregates can vary widely from as small as 100 μm to as large as several millimeters.

Conclusion

Our experimental data shows that the growth of suspension cells of "*Yin-hong*" grape are relatively sensitive to exogenous growth regulators, different concentrations of 6-BA and NAA had significant effects on cells' number, PCV, DCW and viability. Suitable levels of growth regulators are critical for rapid growth of grape suspension cells .The optimum suspension culture medium of "*Yin-hong*" grape is determined as B5 + 250 mg/L casein hydrolysate + 1.5 mg/L 6-BA + 1.5 mg/L NAA + 30 g/L sugar. Within this optimum medium, 1) the maximum number of suspension cells after the logarithmic growth phaseis 34.78×10^8 / mL; 2) the highest cell viability reached 86.45%; 3) DCW reached 3.84 g/L; 4) PCV reached 0.092 mL/mL after eight days cultivating. A system of rapid growth of suspension cell of "*Yinhong*" grape has been established, which can lay a good foundation for further research of anthocyanin biosynthesis and application.

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References

Baldi, A. and V.K. Dixit. 2008. Yield enhancement strategies for artemisinin production by suspension cultures of *Artemisia annua*. *Bioresource Technoli*, 99: 4609-4614.

Botha, F.C. and M.M. O'Kennedy. 1998. Carbohydrate utilization by cell suspension cultures of *Phaseolus vulgaris*. *Plant Physiol*., 102: 429-436.

Cai, Z.Z., H. Riedel, N.M.M.T. Saw, O. Kütük, I. Mewis, H. Jäger, D. Knorr and I. Smetanska. 2011. Effects of pulsed electric field on secondary metabolism of *Vitis vinifera* L. cv. Gamay Fréaux suspension culture and exudates. *Appl. Biochem. Biotechnol*., 164: 443-453.

Deroles, S. 2009. Anthocyanin biosynthesis in plant cell cultures: a potential source of natural colour ants. In: *Anthocyanins*. (Eds.): Winefield, C., K. Davies & K. Gould. Springer Publishers, The New York, pp.108-167.

Fontes, N.; S. Delrot and H. Gerós. 2010.A method for the isolation of protoplasts from grape berry mesocarp tissue. *Recent Pat. Biotechnol*., 4(2): 125-129.

Garshasbi, H., M. Omidi, S. Torabi and D. Davodi. 2012. The study of phytohormones and explants on callus induction and regeneration of sainfoin (*Pnobrychis sativa*). *Pak. J. Agri. Sci*., 49: 319-322.

Kanabus, J., R.A. Bressan and N.C. Carpita. 1986. Carbon assimilation in carrot cells in liquid culture. *Plant Physiol*., 82: 363-368.

Karwasara, V.S., R. Jain, P. Tomar and V.K. Dixit. 2010. Elicitation as yield enhancement strategy for glycyrrhizin production by cell cultures of *Abrusprecatorius* Linn. *In Vitro Cell Dev. Biol. Plant*, 46: 354-362.

Kharenko, O.A., L.I. Zaharia, M. Giblin, V. Cekic, D.C. Taylor, C.D. Palmer, S.R. Abrams and M.C. Loewen. 2011. Abscisic acid metabolism and lipid accumulation of a cell suspension culture of *Lesquerellafendleri*. *Plant Cell Tiss. Org. Cult.*, 105: 415-422.

Pasqua, G., B. Monacelli, N. Mulinacci, S. Rinaldi, C. Giaccherini, M. Innocenti and F.F. Vinceri. 2005. The effect of growth regulators and sucrose on anthocyanin production in *Camptothecaacuminata* cell cultures. *Plant Physiol. Biochem*., 43: 293-298.

Qu, J.G., W. Zhang and X.J. Yu. 2011. Instability of anthocyanin composition under different subculture conditions during long-term suspension cultures of *Vitisvinifera* L. var. Gamay Fréaux. *Chinese Journal of Biotechnology*, 27(11): 1613-1622.

Rao, S.R. and G.A. Ravishankar. 2002. Plant cell cultures: Chemical factories of secondary metabolit. *Biotechnol. Adv*., 20: 101-153.

Ravishankar, G.A. and L.V. Venkataraman. 1993. Role of plant cell culture in food biotechnology: current trends, limitationsand future prospects. In: *Plant biotechnology: commercial prospects andproblems*. (Eds.): Prakash, J. and R.L.M. Pierik. Oxford IBH Press, The New Delhi, pp. 255-274.

Rontein, D., M. Dieuaide-Noubhani and E.J. Dufourc. 2002. Stability of central metabolism and flexibility of anabolic pathways during the growth cycle of tomato cells. *J. Biol. Chem.*, 277: 43948-43960.

Satdive, R.K., D.P. Fulzele and S. Eapen. 2007. Enhanced production of azadirachtin by hairy root cultures of *Azadirachtaindica* A. Juss by elicitation and media optimization. *J. Biotechnol.*, 128: 281-289.

Saw, N.M.M.T., H. Riedel, Z.Z. Cai, O. Kütük and I. Smetanska. 2012. Stimulation of anthocyanin synthesis in grape (*Vitisvinifera*) cell cultures by pulsed electric fields and ethephon. *Plant Cell Tiss. Org. Cult.*, 108: 47-54.

Szabados, L., L.A. Mroginski and W.M. Roca. 1993. Suspension escelulares: descripción, manipulacióny aplicaciones. In: *Cultivo de Tejidos en la Agricultura:Fundamentosy Aplicaciones*. (Eds.): Roca, W.M. and L.A. Mroginski. Cali, CIAT, pp. 174-210.

Víctor, M. Loyola-Vargas and F. Vázquez-Flota. 2006. Plant Cell Culture Protocols, 2nd ed. In: *Methods in Molecular Biology,* (Ed.): John, M.W. Humam press, The Totowa, pp. 53-56.

Weiler, E.W. 1981. Dynamics of endogenous growth regulators during the growth cycle of a hormone-autotrophic plant cell culture. *Naturwissenschaften*, 68(7): 377-378.

Zha, X.Q., J.P. Luo, S.T. Jiang and J.H. Wang. 2007. Enhancement of polysaccharide production in suspension cultures of protocorm like bodies from *Dendrobiumhuoshanense* by optimization of medium compositions and feeding of sucrose. *Process Biochem.*, 42: 344-351.

Zhao, J.L., L.G. Zhou and J.Y. Wu. 2010. Effects of biotic and abiotic elicitors on cell growth and tanshinone accumulation in *Salvia miltiorrhiza* cell cultures. *Appl. Microbiol. Biotechnol.*, 87: 137-144.

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