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Optimisation of phenolic compound biosynthesis in grape (Bogazkere *Cv.*) callus culture

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The objective of this study was to improve metabolite accumulation of grape cells by manipulating culture and environmental conditions. Response surface methodology (RSM) was used to design and conduct the study with the independent variables being: percent depletion of KH_2PO_4 or NH_4NO_3 and light intensity. Wounded grape leaves (Bogazkere *cv.*) were used for the initiation of callus formation. Grape calli were placed on the modified Murashige and Skoog (MS) media (from 0 to 75% KH_2PO_4 or NH_4NO_3 depleted) under various light regimes (from 30 to 120 µmol m^{-2} s^{-1}), and incubated in a controlled environment for three weeks to stimulate secondary metabolite biosynthesis. The anthocyanin - total phenolic (TP) contents of the calli were determined using chemical analyses and the antioxidant capacity of the cultures were determined by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Higher KH_2PO_4 depletion and 75 µmol m^{-2} s^{-1} light intensity favored the biosynthesis of anthocyanins and the other phenolic compounds and resulted in elevated antioxidant capacity in the cultures. However, an increase in KH_2PO_4 or NH_4NO_3 depletion resulted in a significant decrease in cell weight. Similar to that, increase of light intensity was correlated with decrease of cell weight. Further elevation of light intensity and KH_2PO_4 -NH_4NO_3 depletion was fatal to grape cells and resulted in formation of non-growing watery-brownish cultures. Individual phenolic compounds found in the grape cell cultures were identified using the gas chromatography-flame ionization detector (GC-FID) system. Catechin and epicatechin were identified as the most abundant simple phenolic compounds present in the cultures.

Key words: Antioxidant capacity, callus, grape, phenolic compounds, response surface methodology.

INTRODUCTION

Phenolic compounds have been the subject of numerous scientific reports for their ability to scavenge intra-cellularly produced free radicals. Free radical activity causes uncontrolled cell death, DNA damage, tumor formation and cardiovascular disorders (Fresco et al., 2010; Loke at al., 2010; von Sonntag, 2006). Demand for natural products expanded following epidemiological studies documenting the health promoting properties of phenolic compounds for prevention of disorders related to free radical accumulation in the organisms (Szajdek and Borowska, 2008). Solvent extracts of the plants, fruits, leaves, and seeds are being used to obtain natural food colorants and functional compounds (Wallace and Giusti, 2008) valuable for industrial production of food additives, dietary supplements, functional foods and phyto-tablets. However, economical considerations, geographical limitations, seasonal restrictions and environmental problems (harsh chemical extraction) pushed researchers to seek

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Abbreviations: RSM, Response surface methodology; **HPLC,** high performance liquid chromatography; **NAA,** 1-naphthaleneacetic acid; **BA,** 6-benzylaminopurine; **2,4-D,** 2, 4- dichlorophenoxy acetic acid; **DPPH,** 1-diphenyl-2-picrylhydrazyl; **TP,** total phenolic; **GC,** gas chromatography; **MS,** Murashige and Skoog; **Chs,** Chalcone synthase.

alternative renewable resources for biosynthesis of flavonoids, anthocyanins, and other phenolic com-pounds (Blando et al., 2004; Nicoletti et al., 2008). *In vitro* cell cultures may pose an alternative and valuable tool for the production of bioactive compounds. Plant calli and cell cultures exhibit many advantages over plant tissues in obtaining of aforementioned compounds such as ease of extraction, manipulation of direction of biosynthesis, and scalable production. Secondary metabolite production using callus cultures has been reported in *Prunus cerasus, Daucus carota, Cleoma rosea, Rosmarinus officinalis, Vaccinium macrocarpon* (Blando et al., 2004; De Bellis et al., 2005; Glagben et al., 1992; Simoes et al., 2009; Kuhlmann and Rohl 2006; Mathavi et al., 1995).

Grape is one of the most potent fruits suitable for initiation of cell culture system for the production of phenolics and it displays high antioxidant activity with a wide range of secondary metabolites. Bogazkere cv. is a predominant grape variety growing in Southeastern and Eastern regions of Turkey with stringent taste and high phenolic content which makes it a good candidate to initiate cell cultures for production of secondary metabolites. Manipulation of medium composition, light intensity, temperature, growth regulators, and presence - concentration of intermediaetic compounds may affect the productivity and viability of *in vitro* cultures (Georgiev et al., 2008). Optimization of the callus growth conditions is one of the prerequisites for the stimulation of secondary metabolite biosynthesis and development of protocols allowing establishment of viable cultures. Response surface methodology (RSM) is being used to design experiments to meet the best operation conditions to achieve acceptable product yields, improve efficiency - profitability, while consuming limited time and amount of energy. The aim of this investigation was to determine the best medium composition regarding KH_2PO_4 or NH_4NO_3 concentrations and environmental conditions by using RSM skills to induce secondary metabolite biosynthesis in grape callus. The anthocyanin accumulation, phenolic content and antioxidant activity were investigated in the grape callus grown in various media and under different light regimes. The medium composition and light intensity were modified to improve the biosynthesis of the secondary metabolites by using a three factorial experimental design.

MATERIALS AND METHODS

Materials

High performance liquid chromatography (HPLC)-grade ethanol and methanol, HCl, ammonium nitrate, potassium chloride, sodium chloride, sodium carbonate, sodium acetate, Folin-Ciocalteau reagent, were purchased from MERCK Chemical Company, Germany. Gamborg-B5 medium, 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BA), 2,4- dichlorophenoxy acetic acid (2,4-D), reagent-grade gallic acid, were purchased from Sigma Chamical Co. 1,1-diphenyl-2-picrylhydrazyl (DPPH) was obtained from Fluka (Chemie AG, Bucks).

Grape callus induction and secondary metabolite biosynthesis

The grapevine leaves of *Vitis vinifera* L. cv. Bogazkere used for callus formation were taken from long-term in vitro cultures established and maintained according to the method described by Ozden et al. (2009). Leaf discs from grapevine (*V. vinifera*) cv. Bogazkere were excised and placed abaxial side up on solid Gamborg B-5 (Gamborg et al., 1968) medium with vitamins in petri dishes. Plant growth regulators, 0.2 mg/L BA and 0.02 mg/L 2, 4-D were added to the medium to reinforce callus formation (Keller et al., 2000). The pH value of the medium was adjusted to 5.8 prior to autoclaving (121°C, 1 kg cm^{-2} for 20 min). Calli were initiated from young leaf explants and incubated at 24 ± 1°C in darkness in controlled growth room. After four weeks, calli were separated from the explants and subcultured to fresh media for four weeks more for callus proliferation under 16 h photoperiod at 24±1°C. Afterwards, the calli were submerged and cultivated in Petri dishes with modified Murashige and Skoog (MS) media supplemented with 1 mg/L NAA and 0.1 mg/L BA for the induction of anthocyanin and the other phenolic compounds' biosynthesis. After three weeks of culture, calli were harvested, pooled, and used to obtain acidified methanol extracts of callus. Modifying the medium composition and light intensity on the biosynthesis of the secondary metabolites in grape callus was evaluated according to the response surface, 3-level factorial design (Table 1) with two variables; percent depletion (0 to 75%) of KH_2PO_4 and NH_4NO_3 in the MS and light intensity (30 to 120 µmol m^{-2} s^{-1}) based on the results obtained in the preliminary studies.

Extraction of phenolics from callus

For sample extraction, calli were homogenized in an ice cold blender and 10 g of the homogenate was macerated in 20 mL of methanol containing 20 µl conc. HCl and was left incubated in darkness overnight at 4°C. Then the extract was filtered over Whatman No. 1 paper under vacuum, and fresh acidified methanol was used to re-extract the residue until it became colorless. The alcohol in the extract was evaporated using rotary evaporator.

Total phenolic (TP) analysis

TP content of the callus were estimated according to the method of Slinkard and Singleton (1977) with some modifications. Samples of 0.03 mL were introduced into test tubes, 2.370 mL distilled water and 0.15 mL Folin-Ciocalteu's reagent was added and the tubes were vortexed vigorously. After 8 min, a 0.45 mL of saturated Na_2CO_3 was added to each tube, and then each mixture was vortexed again and allowed to stand for 30 min at room temperature. Absorbance was measured at 750 nm. Gallic acid was used to create a standard curve. The results were expressed as mg gallic acid equivalent.

Total anthocyanin analysis

Anthocyanin content of the callus was determined by the pH differential method described by Giusti and Wrolstad (1996) using a ultraviolet (UV)-vis spectrophotometer (Model U-2000, Hitachi). Absorbance of the samples in 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5) were measured at 520 and 700 nm. Anthocyanin content was determined using the equation: $A = (A_{\lambda 520} - A_{\lambda 700})_{pH\ 1.0} - (A_{\lambda 520} - A_{\lambda 700})_{pH\ 4.5}$ with a molar extinction coefficient of 28,000. Results were expressed as mg of

Table 1. Experimental conditions of callus growth treatments for phenolic compounds.

Run Number	Independent variable				
	Depletion		Light Intensity		MS Media Component
	Coded level	Actual level	Coded level	Actual level	Categorical factor
1	-1	0.0	0	75	NH_4NO_3
2	0	37.5	0	75	KH_2PO_4
3	1	75.0	0	75	KH_2PO_4
4	-1	0.0	1	120	NH_4NO_3
5	0	37.5	0	75	NH_4NO_3
6	0	37.5	0	75	KH_2PO_4
7	0	37.5	0	75	NH_4NO_3
8	-1	0.0	-1	30	NH_4NO_3
9	0	37.5	1	120	NH_4NO_3
10	0	37.5	0	75	KH_2PO_4
11	-1	0.0	0	75	KH_2PO_4
12	0	37.5	1	120	KH_2PO_4
13	-1	0.0	1	120	KH_2PO_4
14	1	75.0	1	120	KH_2PO_4
15	0	37.5	0	75	NH_4NO_3
16	0	37.5	0	75	NH_4NO_3
17	1	75.0	1	120	NH_4NO_3
18	1	75.0	0	75	NH_4NO_3
19	-1	0.0	-1	30	KH_2PO_4
20	0	37.5	-1	30	NH_4NO_3
21	1	75.0	-1	30	NH_4NO_3
22	1	75.0	-1	30	KH_2PO_4
23	0	37.5	0	75	KH_2PO_4
24	0	37.5	0	75	KH_2PO_4
25	0	37.5	-1	30	KH_2PO_4
26	0	37.5	0	75	NH_4NO_3

malvidin-3-O-glucoside equivalent in per kg fresh fruit (Wrolstad, 1976).

Antioxidant capacity determination

Free radical scavenging activity (antioxidant capacity) of the samples were determined by the DPPH assay with minor modifications as described by Blois (1958). A 0.1 mM solution of DPPH in methanol was prepared. A 0.1 mL of various concentrations of the samples diluted in methanol was added to 2.9 mL of DPPH solution. The decrease in absorbance at 517 nm was measured after 30 min of incubation at room temperature. Radical scavenging capacity of each sample was expressed as percent DPPH radical scavenging effect using the following equation.

DPPH scavenging effect (%) = $[(A_c-A_s)/A_c \times 100]$

Where, A_c is the absorbance of the control in ethanol, and A_s is the absorbance of the sample. Assays were carried out in triplicates.

Gas chromatography (GC) analysis

The phenolic compounds found in grape calli were identified and quantified using a Thermo Quest, CE Instrument trace GC equipped with a flame ionization detector (GC-FID). Phenolic compunds were separated by using a ZB-5 (Zebron) capillary GC column (30 m x 0.25 mm x 0.25 µm film thickness). The method described by Soleas et al. (1997) was essentially used for solid phase extraction and derivatization. One µl of solution injected to GC for analysis. Similar GC temperature program, carrier gas flow rate, and split ratio were used as mentioned in an earlier study (Soleas, Diamandis, Karumanchiri and Goldberg, 1997). Authentic standards were used to identify phenolic compounds according to their retention times. Quantification was accomplished by using fisetin (10mg/L) as internal standard. The standards used in this study were kindly provided either by Dr. Geza Hrazdina (Cornell University) or obtained from the Sigma company.

Statistical analysis

The response surface, 3-level factorial design was used to determine the influence of modifying the medium composition and light intensity on the biosynthesis of the secondary metabolites in grape callus. The responses were analyzed using the software trial version Design-Expert® V.7.1.6, StatEase, Inc., (MN, USA). The effects of the independent variables on the properties of the callus

Table 2. Experimental responses from the callus extracts treatments.

Run	Response			
	Anth.	TP	AC	CW
1	34.5±0.2	1690±12	35±1	6.2±0.2
2	49.4±0.3	3045±24	66±2	1.5±0.1
3	68.9±0.2	8021±72	91±3	1.6±0.1
4	31.5±0.2	1455±20	31±2	6.4±0.2
5	40.1±0.3	2618±26	54±1	4.0±0.2
6	49.1±0.2	3020±20	66±2	1.4±0.1
7	40.5±0.2	2590±29	55±3	4.0±0.2
8	24.8±0.1	1315±13	31±2	6.4±0.3
9	39.7±0.2	1920±14	49±1	4.0±0.1
10	49.6±0.2	3082±23	66±1	1.5±0.1
11	34.5±0.1	1690±15	34±2	6.2±0.2
12	43.5±0.2	2151±21	52±1	1.9±0.1
13	31.5±0.1	1455±11	31±1	6.4±0.3
14	54.6±0.2	7650±31	83±3	1.8±0.1
15	40.6±0.1	2580±32	55±2	4.0±0.2
16	40.8±0.1	2628±19	55±2	4.0±0.2
17	43.2±0.1	2650±32	70±1	4.1±0.1
18	51.0±0.1	3610±23	73±2	4.1±0.1
19	24.8±0.1	1315±14	31±2	6.5±0.2
20	36.5±0.1	1725±16	48±1	4.4±0.1
21	41.4±0.1	2511±23	68±2	4.2±0.1
22	50.7±0.2	6410±42	75±2	4.1±0.2
23	49.6±0.3	3051±26	64±2	1.4±0.0
24	49.7±0.3	3057±32	65±3	1.4±0.0
25	41.1±0.1	2015±27	51±2	4.3±0.2
26	39.4±0.1	2604±22	55±2	4.0±0.1

Anth, anthocyanin content of callus (mg/kg); **TP**, (mg/kg) total phenolics of callus;
AC, (% Inhibition), antioxidant capacity of callus extracts; **CW**, (g) cell weight of callus.

were assessed with a design consisted of 26 treatments. A quadratic model was fitted to the experimental data to predict the dependent variable Y (percent depletion of chemical (0-75 %) and light intensity (30-120)):

$$Y = a + b x_1 + c x_2 + d x_1 x_2 + e x_1^2 + f x_2^2$$

Where *a, b, c, d, e, f*, denote the regression coefficients. The three level factorial design model was validated statistically by analysis of variance (ANOVA), calculation and evaluation of the multiple-correlation coefficients, and estimation of the lack of fit.

RESULTS AND DISCUSSION

Secondary metabolite producing grape callus cell lines were placed on MS media containing different concentrations of phosphate and nitrate ions (KH_2PO_4 or NH_4NO_3 concentration was decreased from 0% to 75%), and exposed to various light intensities (30 to 120 μmol m^{-2} s^{-1}). Grape calli were harvested after three weeks of incubation in a tissue culture room and examined for their anthocyanin - total phenolic content, and antioxidant capacity by using chemical experiments. The experimental responses of the light and medium treatments on grape calli are shown in Table 2, while the regression coefficients for each dependent variable (response) as a function of x_1 (% Depletion), x_2 (light intensity), and categoric factor (MS Media Nutrient Composition) are shown in Table 3.

Effect of media composition on secondary metabolite production

The percentage depletion of KH_2PO_4 exhibited highly significant linear positive effect on grape callus anthocyanin content at $p<0.01$. Anthocyanin contents of the grape calli were significantly increased by increasing x_1 and x_2. TP concentrations of the grape calli were drastically increased by increasing all variables mainly x_1 and x_2 while there was a negative quadratic effect of x_2 which was almost negligible. Anthocyanin and TP contents of the plant cells remarkably contribute to their free radical scavenging activity and antioxidant capacity. The antioxidant capacity of the calli was also increased with all the increasing variables (R^2 = 0.9823) except for the slight negative quadratic effect of x_2 which displayed very similar pattern obtained for calli's TP contents. For

Table 3. Regression equations of the models for the anthocyanin total phenolic contents and antioxidant capacity of grape callus grown on different media and ligth regimes.

Response	Equation
Anth.	$\boldsymbol{Y}(KH_2PO_4) = 10.85 + 0.41\boldsymbol{x_1} + 0.58\boldsymbol{x_2} - 5.70\text{x}10^{-4}\,\boldsymbol{x_1x_2} + 7.97\text{x}10^{-5}\,\boldsymbol{x_1^2} - 3.41\text{x}10^{-3}\,\boldsymbol{x_2^2}$
	$\boldsymbol{Y}(NH_4NO_3) = 10.42 + 0.24\boldsymbol{x_1} + 0.57\boldsymbol{x_2} - 5.70\text{x}10^{-4}\,\boldsymbol{x_1x_2} + 7.97\text{x}10^{-5}\,\boldsymbol{x_1^2} - 3.41\text{x}10^{-3}\,\boldsymbol{x_2^2}$
TP	$\boldsymbol{Y}(KH_2PO_4) = -822.14 + 17.27\,\boldsymbol{x_1} + 59.59\boldsymbol{x_2} + 0.08\,\boldsymbol{x_1x_2} + 0.73\,\boldsymbol{x_1^2} - 0.38\,\boldsymbol{x_2^2}$
	$\boldsymbol{Y}(NH_4NO_3) = -572.49 + 26.88\,\boldsymbol{x_1} + 66.36\boldsymbol{x_2} - 0.15\,\boldsymbol{x_1x_2} - 0.014\,\boldsymbol{x_1^2} - 0.42\,\boldsymbol{x_2^2}$
AC	$\boldsymbol{Y}(KH_2PO_4) = 15.05 + 0.62\boldsymbol{x_1} + 0.61\boldsymbol{x_2} + 7.41\text{x}10^{-4}\,\boldsymbol{x_1x_2} + 1.04\text{x}10^{-4}\,\boldsymbol{x_1^2} - 4.00\text{x}10^{-3}\,\boldsymbol{x_2^2}$
	$\boldsymbol{Y}(NH_4NO_3) = 15.91 + 0.44\boldsymbol{x_1} + 0.58\boldsymbol{x_2} + 7.41\text{x}10^{-4}\,\boldsymbol{x_1x_2} + 1.04\text{x}10^{-4}\,\boldsymbol{x_1^2} - 4.00\text{x}10^{-3}\,\boldsymbol{x_2^2}$
CW	$\boldsymbol{Y}(KH_2PO_4) = 8.33 - 0.12\boldsymbol{x_1} + 0.06\boldsymbol{x_2} - 1.67\text{x}10^{-4}\,\boldsymbol{x_1x_2} + 1.12\text{x}10^{-3}\,\boldsymbol{x_1^2} + 3.52\text{x}10^{-4}\,\boldsymbol{x_2^2}$
	$\boldsymbol{Y}(NH_4NO_3) = 7.86 - 0.10\boldsymbol{x_1} + 0.05\boldsymbol{x_2} - 1.67\text{x}10^{-4}\,\boldsymbol{x_1x_2} + 1.12\text{x}10^{-3}\,\boldsymbol{x_1^2} + 3.52\text{x}10^{-4}\,\boldsymbol{x_2^2}$

Anth., Anthocyanin content (mg/kg); **TP**, total phenolic contents (mg/kg); **AC**, antioxidant capacity (% inhibition); **CW,** cell weight (g); x_1, % Depletion (coded values, ranging from -1 to 1, which correspond to 0 to 75 respectively); x_2, light Intensity (coded values, ranging from -1 to 1, which correspond to 30 to 120, respectively).

the anthocyanin contents of the grape calli, the increasing order of magnitude of their effect was $x_2 > x_1$, for the TP contents $x_2 > x_1$, and for the antioxidant capacity $x_1 > x_2$.

The depletion of NH_4NO_3 also significantly contributed to the stimulation of secondary metabolite biosynthesis in grape callus, however at a smaller rate in comparison to what was observed in the calli grown on KH_2PO_4 limited media. The x_1 and x_2 variables affected the metabolite accumulation in grape calli grown on NH_4NO_3 limited media at the same pattern to those of grown on KH_2PO_4 depleted media, although with lower magnitudes as shown in Figures 1 and 2 and Tables 1 and 2, except for TP content. Both x_1 and x_2 exhibited positive influence on anthocyanin - total phenolic contents, and antioxidant capacity (Table 3). However, it should be noted that quadratic effect of x_2 was the sole negative effect on TP content of the cultures obtained from KH_2PO_4 depleted media while x_1 - x_2 interaction, and the quadratic effects of both x_1 - x_2 negatively affected the TP content in calli from the NH_4NO_3 limited media. The F values and R^2 coefficients of the models were above 1 and 0.90, respectively, showing the adequacy of the model (Table 4).

Response surface graphs (Figures 1 and 2) show the influence of light intensity and KH_2PO_4 or NH_4NO_3 depletion on metabolite content and antioxidant activity of grape calli. Optimization of nutrient media composition (concentration and type of nitrate ions, phosphate ions, microelements, carbon source, growth regulators) is one of the most effective ways of the induction of secondary metabolite biosynthesis in various tissue and cell culture systems. Red beet hairy root cultures grown on phosphate and/or nitrate limited media displayed elevated metabolite production potential (Taya et al., 1994; Mukundan et al., 1999; Akita et al., 2002). Our data shows that both depletion of KH_2PO_4 and NH_4NO_3 positively affected the metabolite concentration of calli and antioxidant capacity of their extracts (Figures 1 and 2). The highest anthocyanin - TP contents, and antioxidant activity was detected in the grape calli grown on 75% KH_2PO_4 depleted medium under 75 µmol s^{-2} m^{-1}. Calli grown on 75% KH_2PO_4 depleted media finally displayed 1.35 fold higher anthocyanin content, 2.22 fold higher TP content, and 1.25 fold higher antioxidant capacity in comparison to those of grown on 75% NH_4NO_3 depleted media under the same light regime.

Light dependent secondary metabolite production

Initially, we have cultured wounded grape leaf explants in the dark for callus induction and monitoring possible pigmentation and phenolics accumulation. Supplementation of the MS media with 1 mg L^{-1} NAA and 0.1 mg L^{-1} BA was necessary to induce callus formation and growth. Callus induction from grape leaves was accomplished in the dark although there was no visible anthocyanin accumulation in grape calli (Figure 3a). We have applied different light regimes (from 30 to 120 µmol s^{-2} m^{-1}) to the grape calli obtained from the leaf explants cultured in the dark for the stimulation of biosynthesis of anthocyanins and the other phenolics. On transferring callus cultures to light in production medium significant secondary metabolite production was observed (Figure 3b). Highest metabolite accumulation was detected on the 21st day (data not shown) and thus a three week time period was selected for the evaluation of the light effect on the natural compound accumulation in grape calli. Increasing light intensity initially favored the anthocyanin and TP accumulation that brought about a significant increase in antioxidant capacity (Figures 1 and 2). However, further elevation of light intensity resulted in a reduction of those metabolites' concentration in the grape cells. Light-induced secondary metabolite production in plant cells is species-specific and significantly affected by light source, intensity and duration. Light-induced secondary metabolite production was reported in callus cultures of many species such as *Fragaria ananassa* (Nakamura et al.,

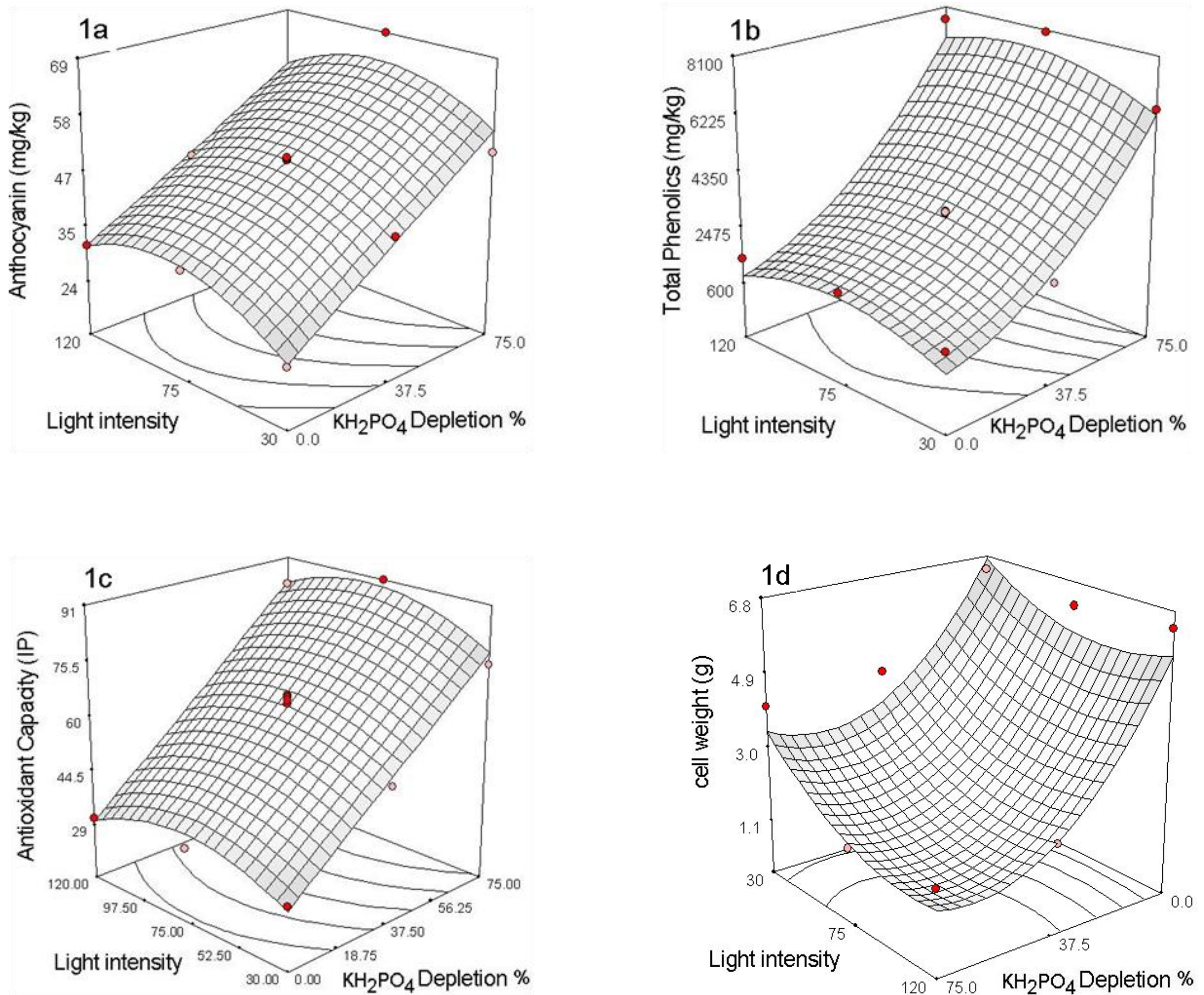

Figure 1 Three dimensional plot depicting 1a) anthocyanin accumulation, 1b) total phenolic compound concentration, 1c) antioxidant capacity, 1d) cell weight as a function of light intensity and KH_2PO_4 depletion.

1999), *Prunus cerasus* L. (Blando et al., 2004), *Vaccinium macrocarpon* Ait (Madhavi et al., 1995), *Centaurea cyanus* (Kakegawa et al., 1987), and *Eleutheretococcus senticosus* (Shohael, 2006). According to the results obtained in this study, exposure of calli to light strongly induced anthocyanin and phenolics biosynthesis that is linked to increased anti-oxidant capacity and the highest metabolite production was detected in grape calli grown under 75 µmol s^{-2} m^{-1} (Figures 1 and 2). It should be noted that elevation of light intensity to 120 µmol s^{-2} m^{-1} causes a significant reduction in anthocyanin - phenolics accumulation and a decrease in antioxidant activity in grape cells. It was observed that light intensities above 75 µmol s^{-2} m^{-1} impair cell formation and development which could be linked to callus vitrification and a decrease in metabolite accumulation by affecting the viability of calli. Similar results were obtained by Chan et al. (2010). They found that *Melastoma malabathricum* cell cultures display highest anthocyanin accumulation when exposed to moderate light intensities (301 to 600 lux). In contrast, a significant decrease in measurable antho-cyanin concen-tration was observed in the calli grown under higher light intensity (900 to 1200 lux). Chalcone synthase (Chs) gene is one of the key genes controlling the phenyl-propanoid pathway that is responsible for anthocyanin biosynthesis and accumulation of Chs mRNA transcripts is closely related to elevation of anthocyanin concen-tration within the cell. Parsley Chs gene's promoter region contains several *cis*- acting elements responsible

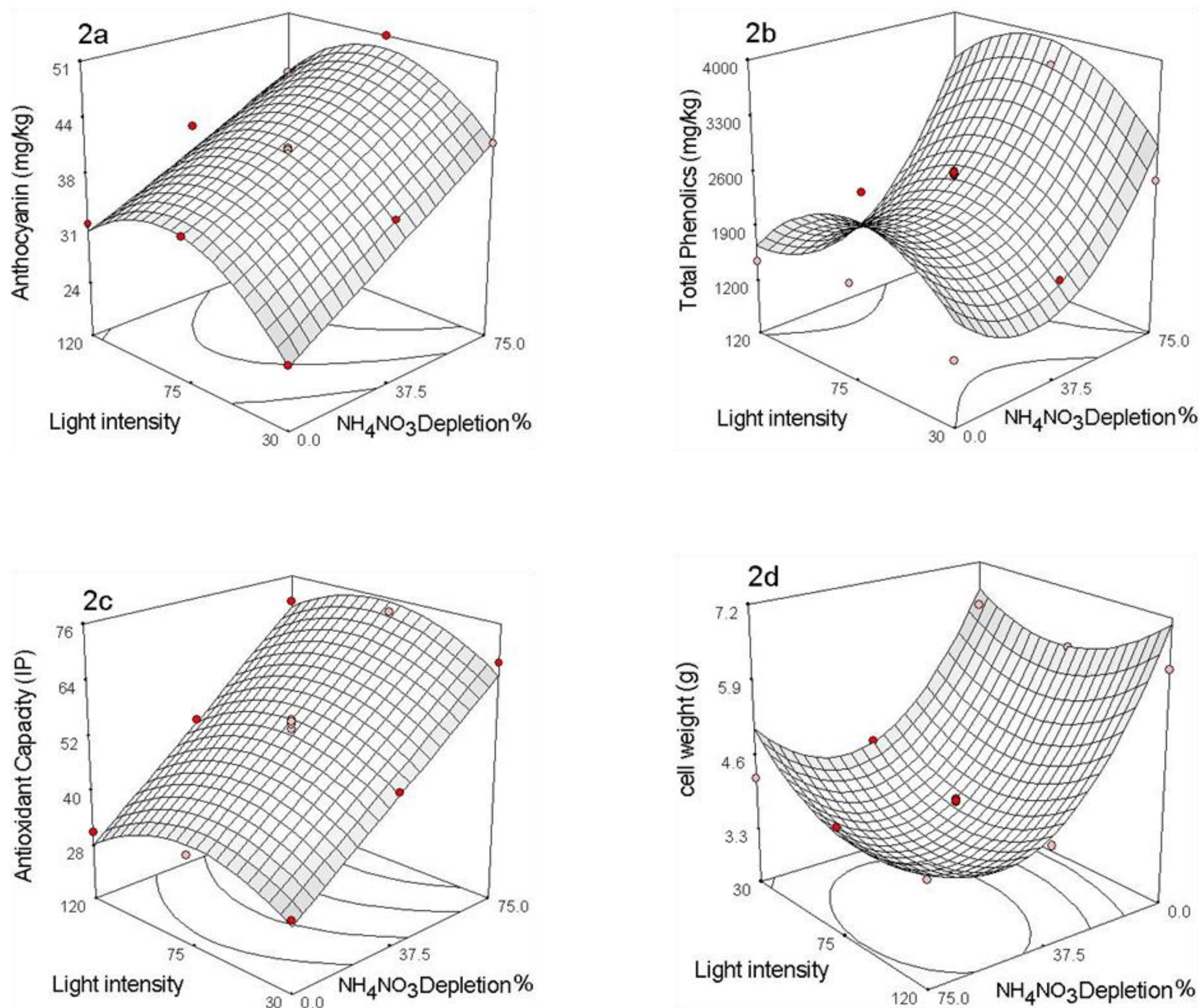

Figure 2. Three dimensional plot depicting 2a) anthocyanin accumulation, 2b) total phenolic compound concentration, 2c) antioxidant capacity, 2d) cell weight as a function of light intensity and NH_4NO_3 depletion.

for light-mediated activation of Chs mRNA transcription (Schulze-Lefert et al., 1989). However, Chs transcription is strongly and exclusively stimulated by only low fluence and short wavelength light (Chappel and Hahlbrock, 1984). Arabidopsis cells respond to low level of UV-light irradiance by stimulating the Chs transcription without apparent oxidative damage (Christie and Jenkins, 1996; Frohnmeyer et al., 1997) light irradiation may cause an increase in the cellular anthocyanin concentration whereas cell growth might be suppressed (Zhang et al., 2002). Over exposure of plant cells to light might cause suppression of Chs transcription and an oxidative stress leading to oxidative damage (Green and Fluhr, 1995) those might be the major factors causing a reduction in anthocyanin accumulation observed in our study. Therefore, under the light and nutrient conditions applied in this study, the best growth conditions to obtain highest secondary metabolite by using grape cell culture *cv.* Bogazkere were as follows, 75 µmol $m^{-2}s^{-1}$ light intensity with 75% KH_2PO_4 depleted MS media.

Effects of media composition and light intensity on the cell weight

Grape cell proliferation was determined for all the calli placed on various KH_2PO_4 or NH_4NO_3 depleted media used in this study under different light regimes and expressed on the gram basis. Equal amount of grape callus (100 mg) was located on the media for culture initiaton and further analysis of the effect of KH_2PO_4 or NH_4NO_3 concentrations on the cell growth under certain

Table 4. Statistical evaluation and validation tests applied to regression equations.

Parameter	Regression	Residual	Total	R^2	Lack of Fit	Pure error
Anth.						
Sum of squares	2197.06[a]	104.62	2301.68	0.9545	102.80[a]	1.82
Freedom degrees	8	17	25	-	9	8
Mean squares	274.63	6.15	-	-	11.42	0.23
F value	44.63	-	-	-	50.32	-
TP						
Sum of squares	$7.21x10^7$ [a]	$6.06x10^6$	$7.82x10^7$	0.9225	$6.06x10^6$ [a]	3538
Freedom degrees	8	17	25	-	9	8
Mean squares	$9.01x10^6$	$3.56x10^5$	-	-	$6.73x10^5$	442.25
F value	25.30	-	-	-	1521.21	-
AC						
Sum of squares	6860.71 [a]	123.79	6984.50	0.9823	117.59 [a]	6.20
Freedom degrees	8	17	25	-	9	8
Mean squares	857.59	7.28	-	-	13.07	0.78
F value	117.77	-	-	-	16.86	-
CW						
Sum of squares	73.44 [a]	6.49	79.93	0.9189	6.48 [a]	$1.82x10^{-3}$
Freedom degrees	8	17	25	-	9	8
Mean squares	9.18	0.38	-	-	0.72	$2.28x10^{-4}$
F value	24.06	-	-	-	3166.96	-

Anth., Anthocyanin (mg/kg); TP, total phenolic (mg/kg); AC, antioxidant capacity (% inhibition); CW, cell weight (g). [a]Significant for α = 0.01.

light intensities. Cell weight was determined after three weeks of time period. KH_2PO_4 or NH_4NO_3 depletion deteriorated cell proliferation and negatively correlated with the culture growth. 75% depletion of KH_2PO_4 resulted in almost four times less cell growth weight (from 6.8 to 1.8 g) compared to that of control samples (0% KH_2PO_4 depletion) under highest light regime (120 µmol $m^{-2}s^{-1}$) (Figures 1 and 2). Similarly, grape cells grew approximately two times less on 75% NH_4NO_3 depleted media in comparison to the control samples growing under the same light regimes. According to the results obtained in this study; even though depletion of KH_2PO_4 or NH_4NO_3 positively contributes to accumulation of phenolic compounds, optimisation studies indicated a critical balance zone shown in contour plots (Figure 4a, b) for each of KH_2PO_4 or NH_4NO_3 depletion for the most optimum production efficiency for certain phenolic compounds while keeping in mind callus viability and cell weight which are also two important factors determining the best conditions of cellular production of industrially important natural compounds.

Light intensity was also another significant determinant of total concentrations of the analyzed compounds and final cell weight. Higher light intensities (75 µmol $m^{-2}s^{-1}$) remarkably stimulated anthocyanin - TP biosynthesis in comparison to that of grown under lower light regimes (30 µmol $m^{-2}s^{-1}$). Results indicated detrimental effect of light intensities above 100 µmol $m^{-2}s^{-1}$ on the cell growth and brought about development of smaller cultures that was considered inefficient regarding metabolite accumulation on the basis of measured cell weight.

In conclusion, the grape callus cultures obtained with the optimal conditions displayed a higher TP and anthocyanin content together with greater antioxidant capacity. Cell viability and weight was limiting factor for our study interfering the potential increment of light intensity or further depletion of media components, namely KH_2PO_4 or NH_4NO_3. Healthy and viable callus formation and growth was the primary concern for establishing cell cultures and concluding most optimal conditions those will be recommended for bioproduction of natural compounds under mentioned conditions. Optimisation plot containing variables and responses are shown in Figure 4a, b. An increase in light intesity and depletion of both media components induced secondary metabolite production and intersection of those responses with cell weight determined the optimal contour plot indicating optimal zone showing most optimum variable factors for the best culture growth and secondary metabolite biosynthesis. Significant variables were utilised to generate response surface plots and their contours against each response. A multi-response optimization technique was employed to determine the optimum combination of KH_2PO_4 or NH_4NO_3 concentra-

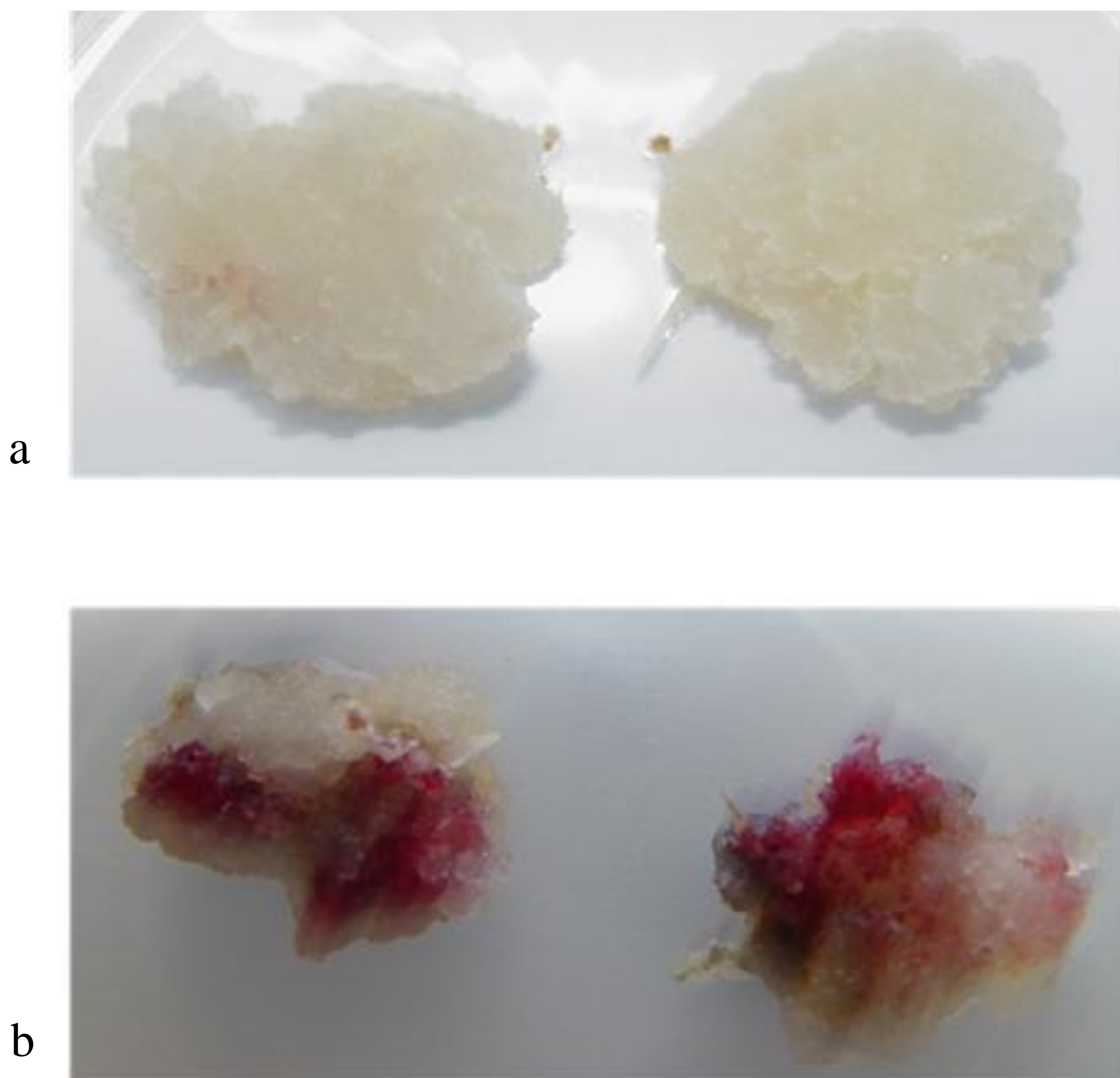

Figure 3. a, Grape calli grown on MS media in the dark; b, pigmentation in grape calli when exposed to light (75 μmol m^{-2} s^{-}

tions and light intensity for the most optimum conditions of callus growth accumulating anthocyanin and phenolic compounds. The main criteria constraint optimization were growth and viability to be as high as possible with high antioxidant capacity, anthocyanin and phenolic compound content combined with improved concentrations of individual natural compounds as determined by GC-FID system.

The optimum culture growth con-ditions can then be determined by superimposing the contour plots of relevant responses The contour plots indicated that 30% depletion of KH_2PO_4 or 50% depletion of $NH4NO_3$ from media exposed to 65 or 75 μmol $m^{-2}s^{-1}$, respectively, light intensities represented the best growing conditions for the grape cultures.

Identification of ındividual phenolic compounds found in callus cultures

One of the main objectives of this study was to improve the concentrations of industrially important phenolics found in grape cultures as they are beneficial to human health and might be used as functional food ingredients.

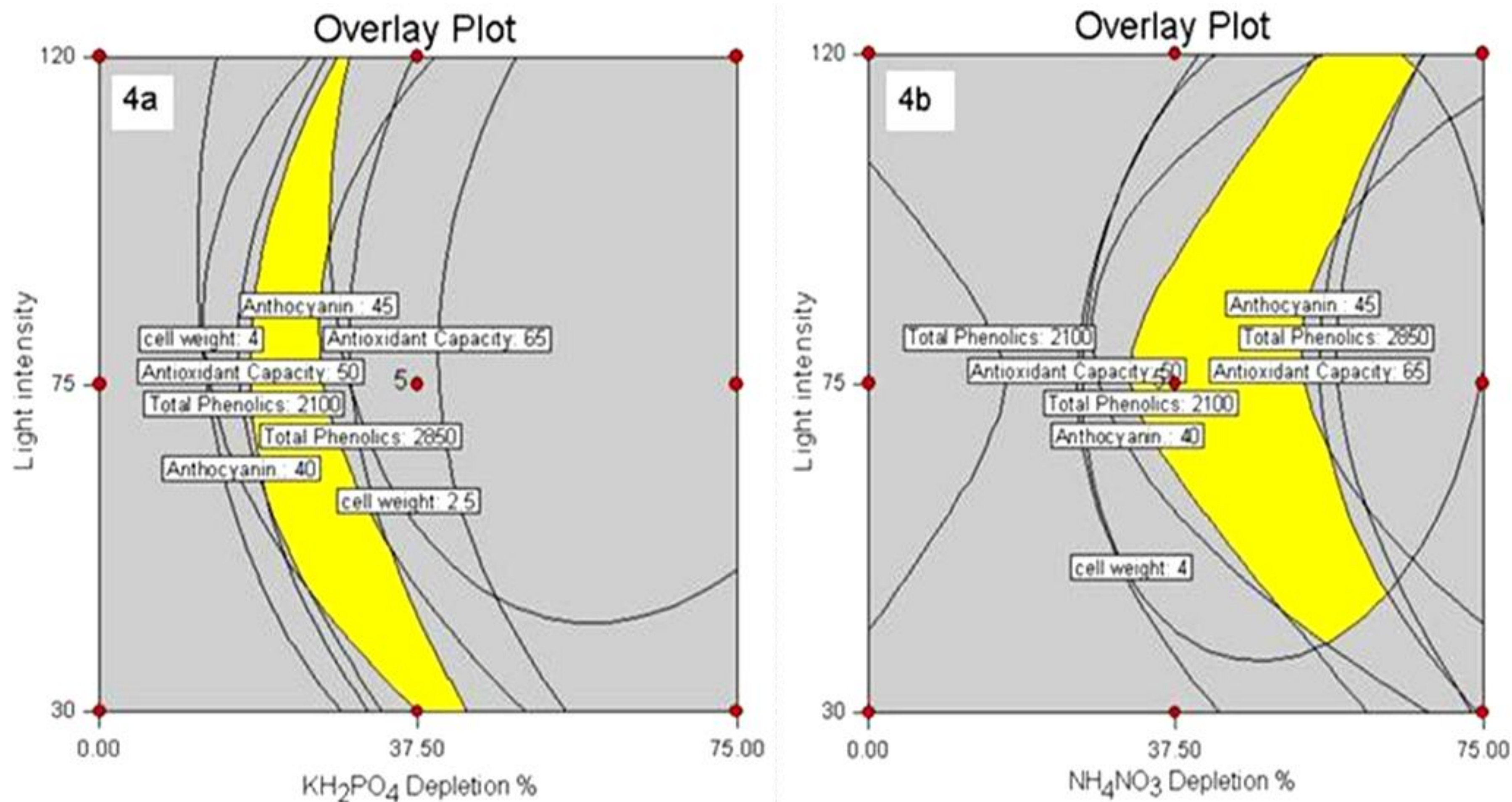

Figure 4. Contour plots showing the effects of light intensity - KH_2PO_4 depletion (4a) and light intensity-NH_4NO_3 (4b) on the variables.

Amount of phenolic compounds found in grape cell cultures were analyzed and individually identified by using a GC coupled to a FID detector system. We analysed the cell cultures grown on 37.5% KH_2PO_4 depleted media exposed to increasing light intensities from 0 to 75 µmol $m^{-2}s^{-1}$. Six simple phenolic compounds were identified by using GC-FID system, namely gentisic acid, gallic acid, epicatechin, vanillin, hesperidin and catechin as shown in GC chromatogram (Figure 5). The amount of identified phenolic compounds increased parallel to increasing light intensities. The most prevelant phenolic compounds was catechin and its amount increased from 29.40 to 137.80 (mg/kg) with the elevation of the light intensity up to 75 µmol $m^{-2}s^{-1}$. The amounts of the identified natural compounds were shown in **Table** 5.

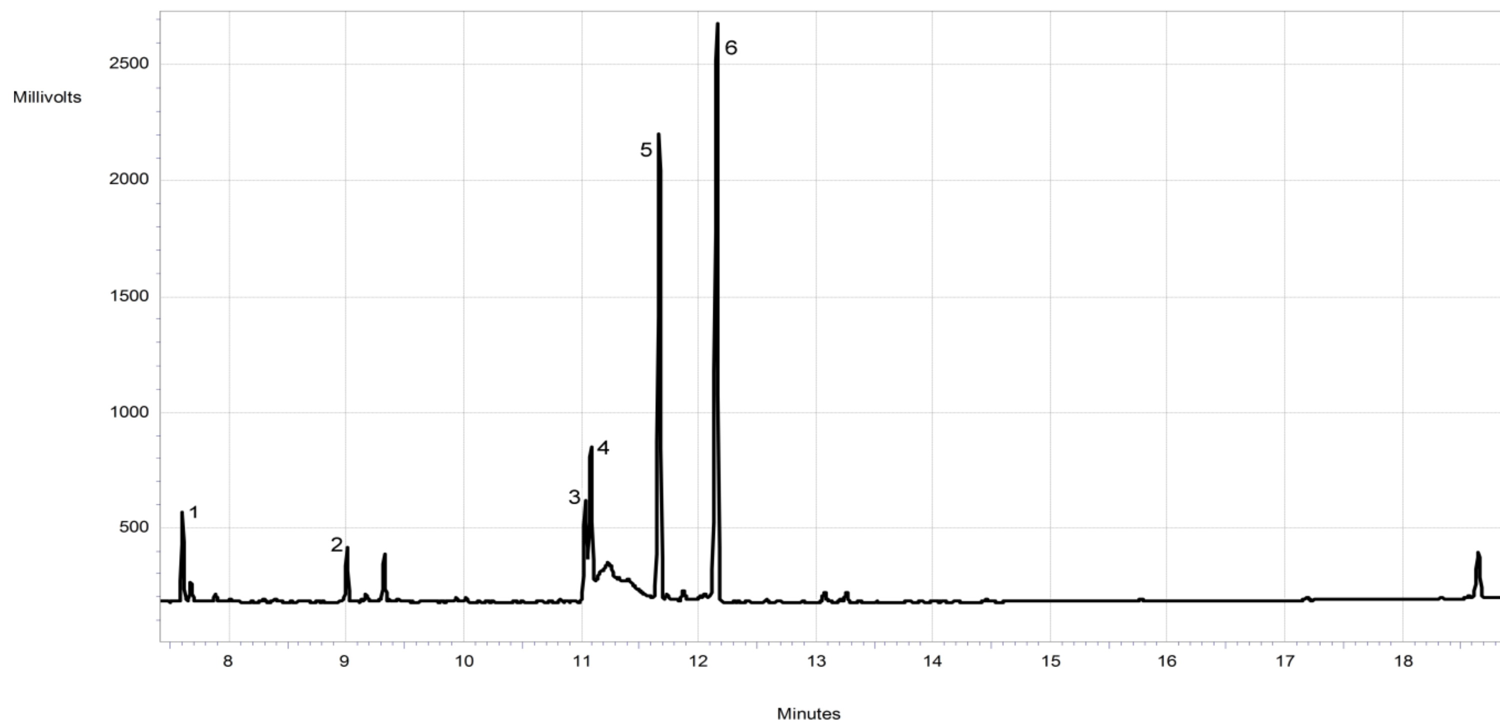

Figure 5. Simple phenolic compounds determined using GC-FID system. 1, Gentisic acid; 2, gallic acid; 3, epicatechin; 4, vanillin; 5, hesperidin; 6. catechin.

Table 5. Simple phenolic compounds detected by GC-FID system and concentration of phenolic compounds at different light intensities.

Simple phenolic compound (mg/kg)	Light Intensity (µmol $m^{-2}s^{-1}$)		
	0	**37.5**	**75.0**
Gentisic acid	0.6	3.21	5.2
Gallic acid	7.2	12.09	16.13
Epicatechin	13.17	18.26	21.54
Vanillin	0.46	6.17	18.5
Hesperidin	0.24	0.43	0.59
Catechin	29.4	92.4	137.8

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