Improvement of Phenolic Antioxidant-linked Cancer Cell Cytotoxicity of Grape Cell Culture Elicited by Chitosan and Chemical Treatments

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Abstract. Grape (Vitis vinifera cv. Pok Dum) is a rich source of health relevant phenolic antioxidants and can be targeted to mitigate chronic oxidative stress commonly associated with noncommunicable chronic diseases (NCDs), such as cancer. Furthermore, improving health relevant phenolic bioactives and associated antioxidant properties of fruits by using chemical elicitation strategy has significant merit. Based on this biochemical rationale, chitosan and other chemical elicitors potassium dihydrogen phosphate (KH₂PO₄), potassium nitrate (KNO₃), sodium selenite (Na₂SeO₃), and aluminum sulphate [Al₂(SO₄)₃] were targeted to improve phenolic bioactive profiles and associated antioxidant and anticancer properties of cultured grape cells grown for 28 days. After chemical elicitor treatments, phenolic content, resveratrol content, antioxidant activity, phenylalanine ammonia-lyase (PAL) enzyme activity, and cytotoxicity (cell inhibition) against cancer cell lines of cultured grape cells were investigated using in vitro assay models. Overall, stimulation of phenolic bioactives and improvement in associated cytotoxicity against cancer cell lines were found in cultured grape cells after chemical elicitation treatments. Chitosan and other chemical elicitors resulted in lower growth of cultured grape cells; however, they enhanced phenolic biosynthesis on a cell weight basis when compared with the control. Chemical elicitor treatments, such as Na₂SeO₃ (50 mg L⁻¹ and 100 mg L⁻¹) and Al₂(SO₄)₃ (50 mg L⁻¹), resulted in enhanced phenolic content at the end of 14 days of culture (1.7, 1.4, and 1.0-fold increase, respectively). Higher accumulation of resveratrol and higher antioxidant activity with Al₂(SO₄)₃ (50 mg L⁻¹) and Na₂SeO₃ (100 mg L⁻¹) elicitation treatments were also observed. Enhanced phenolic bioactives in cultured grape cells in response to chemical elicitation treatment, such as Na₂SeO₃, also resulted in higher cytotoxicity against different cancer cell lines. Therefore, this study indicates that chemical elicitors, such as Na₂SeO₃ and Al₂(SO₄)₃ as well as chitosan in select doses can be targeted to improve phenolic bioactives and associated antioxidant and anticancer properties in cultured grape cells and such strategy has relevance for wider applications with other phenolic antioxidant-enriched fruits.

Phenolic bioactives from plant-based sources have diverse health relevant biological functions and are gaining increasing attention as food flavorings, to develop functional and pharmaceutical ingredients, and for use in cosmetic industries (He and Yan, 2013). In general, fruits are rich in such phenolic bioactives and especially grape (V. vinifera L.) has higher content of human health relevant bioactive compounds, such as resveratrol (3,5,4’-trihydroxystilbene). Resveratrol is in the stilbene class of compounds that have health relevant functional and pharmacological potentials, such as antioxidant and cellular chemoprotective properties (Giri et al., 2012; Jeandet et al., 2002). Plants synthesize these phenolic bioactives, such as resveratrol as part of natural adaptive responses against abiotic and biotic stresses; however, their production often vary due to geographical locations, environmental conditions, and cross-pollination nature of some plant species.

Previous study has confirmed the aforementioned rationale and reported that the differences in environmental conditions have significant influence in phenolic bioactive content and their profiles (Sae-Lee et al., 2012). Therefore, finding uniform and consistent production of health relevant phenolic bioactives in plants is a significant challenge. In this context, in vitro tissue culture strategy allows for the optimal and consistent production of health-targeted phenolic bioactives (Rao and Ravishankar, 2002). Furthermore, biosynthesis of these health relevant bioactive compounds in controlled cell cultures can be stimulated by using additives, supplements, nutrients, elicitors, and signal compounds that mimic abiotic and biotic stress responses (Zhao et al., 2005).

In this context, coupled to elicitation with natural moieties such as chitosan or their derivatives, the macronutrients such as nitrogen, potassium, and phosphorus not only influence plant primary metabolism but also have a role in biosynthesis of the secondary metabolites, such as phenolic compounds, especially in cultured suspension cells (Narayan and Venkataraman, 2002). Furthermore, a key enzyme of phenylpropanoid pathway, such as PAL, which is related to the biosynthesis of phenolics in plants can be induced by abiotic stresses or by chemical elicitors, such as mineral nutrients and chitosan, that mimic stress responses when applied exogenously (Pereira et al., 2006; Ververidis et al., 2007; Vogt, 2010).

Previously, ammonium nitrate (NH₄NO₃) in basic MS medium favored biomass accumulation and phytochemical production in cultured cells of Withania somnifera (Nagella and Murthy, 2010) and Gymnema sylvestre (Praveen et al., 2011). The highest biomass accumulation, total phenolics, and resveratrol of grape suspension cells were also observed in a medium supplemented with 500 mg L⁻¹ NH₄NO₃ (Sae-Lee et al., 2014), whereas bioactive content was reduced under high NH₄NO₃ concentrations. The modulation of phenolic bioactives can be further coupled to heavy metal stress, such as Na₂SeO₃ and aluminum (Cai et al., 2013; Gomes-Junior et al., 2007; Karimi and Khataee, 2012). Chemical elicitors, such as selenium and aluminum, are known to stimulate plant growth and plant stress tolerance by improving beneficial phenolic bioactives and associated antioxidant activity (Kapoor et al., 2012; Konarska, 2010). However, the safe and human health relevant consumption threshold for potassium, phosphorus, aluminum, selenium, and chitosan is 33.0, 0.0002, 1.0, 0.005, and 15 mg kg⁻¹ d⁻¹, respectively (Agency for Toxic Substances and Disease Registry, 2006; U.S. Department of Commerce National Technical Information Service, 2002).
Objective I  

Plant materials. The red grape cv. Pok Dum targeted in this study is rich in phenolics, especially resveratrol with high antioxidant activity (data not shown). This grape cultivar is also popular in Thailand and was obtained from the Sixty-One Market, Bangkok, Thailand. Young leaf explants from 30-d seedlings were soaked with 70% ethanol for 30 s, then in 5% NaOCl for 3 min, and in 2% NaOCl for 5 min, followed by three sequential rinses for 5 min with sterile distilled water to generate sterile explants for grape cell culture induction.

Callus induction and subculture. To induce callus, the sterile leaf explant excised from ‘Pok Dum’ grape seedlings was cultured in MS medium (Murashige and Skoog, 1962), consisting of agar supplemented with 20 mg L⁻¹ sucrose (as carbon source) and 0.1 mg L⁻¹ 6-benzyladenine. The pH of MS medium was adjusted to 5.8 before autoclaving. The cultured cells were incubated at 25 ± 1 °C with 108 μmol·m⁻²·s⁻¹ light for 16 and 8 h dark photoperiod with the 40 W cool-white fluorescent tubes (Phillips, Kolkata, India). Every 14 d, induced callus was subcultured for producing friable cultured callus cells.

Suspension cultured cells. Suspension cultured cells were initiated from the 30-d-old callus cells of grape ‘Pok Dum’ by transferring 2 g of friable callus cells into a 250-mL erlenmeyer flask containing 50 mL of liquid MS medium consisting of the same medium as the cultured callus cells except no agar. To study cell growth, stimulation of phenolic and resveratrol production in cultured grape cells, five elicitors were chosen, among which four were chemical elicitors, such as KH₂PO₄ (at 200, 500, 1000 mg L⁻¹), KNO₃ (at 500, 1000, 2000 mg L⁻¹), Na₂SeO₃ (at 50, 100 mg L⁻¹), Al₂(SO₄)₃ (at 50, 100 mg L⁻¹), and one natural elicitor, chitosan (at 5, 10, 20 mg L⁻¹). All elicitor treatments were compared with two controls: control 1 (with high 10,000 mg L⁻¹ NH₄NO₃) and control 2 (basal MS media without any treatments) (Table 1). The cultured grape cells were maintained on a rotary shaker at 110 rpm at 25 ± 2 °C for 28 d, before the biochemical analyses.

Determination of cell biomass. The effect of chitosan and other chemical elicitor treatments on cell growth was determined by measuring a change of cell dry weight (biomass) over time. Nine flasks of each treatment were harvested every 7 d and were vacuum filtered through Whatman #1 filter paper. Dry weights (DW) of the treated cells were then recorded after drying at 60 °C in an oven until it reached a constant weight.

Determination of total phenolic and resveratrol content. Dried cells were separated from cultured medium and then ground with 1% (v/v) HCl methanol solution at a ratio of solution to treated sample of 50:1 and steeped for 16 h. Treated samples were centrifuged at 10,000 × g for 10 min, and supernatants were used for determination of total phenolics and resveratrol contents. Total phenolic content was determined by the Folin–Ciocalteu method (Giri et al., 2012) with gallic acid standard curve. A volume of 0.95 of mL distilled water was added to 0.05-mL sample, and then mixed with 5 mL of 10% Folin–Ciocalteu reagent and 4 mL of 7.5% sodium carbonate. The mixture was then vortexed thoroughly. After incubation at room temperature (25 ± 3 °C) for 60 min, the absorbance was recorded at 765 nm, using an ultraviolet-Visible

Table 1. The effect of potassium dihydrogen phosphate (KH₂PO₄), potassium nitrate (KNO₃), sodium selenite (Na₂SeO₃), aluminium sulphate [Al₂(SO₄)₃], and chitosan on dry cell weight of Vitis vinifera cv. Pok Dum cell cultures at 7, 14, 21, and 28 d.

| Treatment               | Conc     | Day 7          | Day 14         | Day 21         | Day 28         |
|-------------------------|----------|----------------|----------------|----------------|----------------|
| KH₂PO₄                  | 200 mg L⁻¹| 41.66 ± 0.71 BC| 51.95 ± 1.06 BA| 45.20 ± 1.84 BB| 38.62 ± 1.05 BD|
|                         | 500 mg L⁻¹| 31.64 ± 0.53 C  | 41.78 ± 1.28 DA| 38.52 ± 1.27 CB| 36.10 ± 1.79 CB|
| KNO₃                    | 500 mg L⁻¹| 31.82 ± 1.44 A  | 33.92 ± 2.56 A  | 32.04 ± 1.11 EA| 25.44 ± 1.18 EB|
|                         | 1000 mg L⁻¹| 28.10 ± 1.75 DA | 30.08 ± 0.19 BA| 23.70 ± 1.72 gB| 21.80 ± 2.51 fB|
| Na₂SeO₃                 | 100 mg L⁻¹| 23.90 ± 1.83 cAB| 24.80 ± 1.57 IA| 25.40 ± 0.53 gA| 21.30 ± 1.50 fB|
|                         | 2000 mg L⁻¹| 20.20 ± 1.85 fA  | 21.10 ± 1.59 jA| 13.20 ± 1.69 jB| 14.60 ± 1.79 iB|
| Al₂(SO₄)₃               | 50 mg L⁻¹ | 32.10 ± 1.90 eC  | 38.20 ± 1.70 eA| 37.10 ± 2.40 eB| 28.60 ± 2.20 dC|
|                         | 100 mg L⁻¹| 42.00 ± 1.70 BB  | 45.00 ± 1.10 IA| 35.10 ± 3.00 dC| 27.40 ± 1.90 fD|
|                         | 200 mg L⁻¹| 25.40 ± 3.80 eC  | 32.30 ± 4.70 fA| 31.00 ± 4.20 eB| 21.43 ± 5.50 fD|
| Chitosan                | 5 mg L⁻¹  | 18.90 ± 2.10 IB  | 21.40 ± 5.70 jA| 21.30 ± 1.20 IA| 19.33 ± 4.60 gB|
|                         | 10 mg L⁻¹  | 25.60 ± 4.40 eC | 29.20 ± 2.70 h  | 28.60 ± 2.00 d | 28.60 ± 2.00 d |
|                         | 20 mg L⁻¹  | 25.80 ± 1.90 DC  | 30.40 ± 2.40 gB | 32.25 ± 2.00 eA| 28.95 ± 1.40 dC|
| Control 1 (NH₄NO₃)      | 500 mg L⁻¹| 60.00 ± 2.80 aB  | 63.40 ± 3.00 aA| 53.40 ± 2.40 aB| 53.40 ± 2.40 aC|
| Control 2 (without elicitors) | 200 mg L⁻¹| 23.80 ± 1.80 eB  | 25.20 ± 2.20 iA| 23.40 ± 1.60 hC| 20.23 ± 1.00 gH|

F-test ** ** ** ** **

CV (%)                   | 4.23     | 3.51           | 3.81           | 4.72           | 4.21           |

t-SD                     | 2.14     | 1.99           | 1.96           | 2.12           | 2.12           |

NH₄NO₃ = ammonium nitrate.

Means of three replicates ± s.d in the same column (a, b, c, d…) or in the same line (A–D) with different letters are significantly (P ≤ 0.05), separated by Duncan’s New Multiple Range Test.

**Significantly different at P = 0.01.
Spectrophotometer (Thermo Fisher Scientific, Madison, WI). Phenolic content was measured based on the gallic acid standard curve (10–50 mg·L⁻¹ gallic acid) and was expressed as mg GAE/g DW of the treated sample.

Trans-resveratrol content in suspended cultured grape cells was determined by high performance liquid chromatography (HPLC) with a reversed phase HPLC Agilent 1200 Series Rapid Resolution LC system (Waldbronn, Germany), equipped with the DAD detector. The column was used was a Zorbax Eclipse XDB-C18 column (150 × 4.6 mm i.d., 5 μm thickness) (Agilent, Santa Clara, CA). Column temperature was maintained at 40 °C. The HPLC condition was based on Sakuta et al. (1987), with the following elution profile: 0 min, 83.5% A, 16.5% B; 13 min, 82.0% A, 18.0% B; 15 min, 82.0% A, 18.0% B; 17 min, 77.0% A, 23.0% B; 21 min, 75.0% A, 25.0% B; 27 min, 68.5% A, 31.5% B; 30 min, 0% A, 100% B. The solvent A was glacial acetic acid in water (52.6:900 v/v), and solvent B was 20% phase A and 80% acetonitrile at the flow rate of 1.0 mL·min⁻¹. Trans-resveratrol identification was carried out by comparing the retention time of pure standard, which was then plotted to a standard calibration curve from 0.005 to 10 mg·mL⁻¹. Samples (100 μL) were directly injected after filtration through a 0.45-μm membrane filter. A photodiode array detector (DAD) was used, and the chromatogram was recorded at 306 nm (Sae-Lee et al., 2014).

Objective II

Determination of the activity of L-PAL. Suspension-cultured cells were harvested for evaluating the activity of the key phenolic biosynthesis relevant enzyme, PAL, based on the method described by Bauer et al. (2011).

Briefly, 1 g of suspension-cultured cells were homogenized in 5 mL of 0.05 M sodium borate (pH 8.8) containing 0.1 g of polyvinylpyrrolidone and 5 μm β-mercaptoethanol, and then centrifuged at 6000 × g and 4 °C, for 15 min. For protein analysis, the supernatant was kept at 4 °C. A volume of 1 mL of supernatant (0.4–2 mg of protein) was mixed with 1 mL of 0.1 M sodium borate (pH 8.0) containing 0.02 M L-phenylalanine, and then incubated at 30 °C for 30 min. The PAL activity was determined based on the formation and detection of trans-cinnamic acid at 290 nm by ultraviolet-Visible Spectrophotometer (Thermo Fisher Scientific, Madison, WI). One unit of enzyme activity was defined as the amount of enzyme needed to form trans-cinnamic acid with 0.01 change of absorbance.

Measurement of antioxidant activity based on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. In this study, DPPH radical assay was modified from Giri et al. (2012) and was used to measure total antioxidant activity. Briefly, DPPH (0.1 mm) was prepared in 80% ethanol (v/v), and 3 mL of stock was mixed with 1 mL of sample extract and placed in the dark (25 ± 2 °C, 30 min). The reduction of absorbance at 517 nm was recorded using the ultraviolet-Visible spectrophotometer (Thermo Fisher Scientific), and the antioxidant activity was expressed in μmol Trolox equivalents (TE)/100 g DW of the sample.

Measurement of antioxidant activity based on 2,2-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS⁺) radical scavenging assay. The determination of total antioxidant activity by ABTS⁺ radical scavenging method was followed using the method described by Giri et al. (2012) with the minor modification. Concentrations of 7.0 μm ABTS and 2.45 μm K₂S₂O₈ were mixed for initiating the production of ABTS⁺ cation (ABTS⁺), and then kept in the dark for 12 h, 22 ± 1 °C. The ABTS solution was then diluted with 80% (v/v) ethanol until the absorbance at 734 nm reached 0.70 ± 0.05. For sample analysis, 4 mL of diluted ABTS⁺ solution was added to 1 mL of methanolic extract and mixed thoroughly. The reaction mixture was allowed to stand (22 ± 1 °C, 6 min, dark), and the absorbance was recorded at 734 nm with an ultraviolet-Visible Spectrophotometer (Thermo Fisher Scientific). A Trolox aliquot was used to develop a 50–500 μm range standard curve. All data for the antioxidant activity were expressed in μmol TE/100 g DW of the sample.

MTT cytotoxicity assay. The inhibitory effect of extracts of grape cell cultures after the five elicitor treatments in different doses against five human cancer cell lines were evaluated: the BT474 breast cancer, Chago-K1 lung cancer, Hep-G2 liver cancer, KATO-III stomach cancer and SW620 colon cancer cell lines were used and compared with normal skin fibroblast cell line, using the MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide)] assay. The conversion of tetrassium salt MTT into a blue colored formazan by the mitochondrial enzyme succinate dehydrogenase was used to quantify cell survival and proliferation by detecting only living cells (Mosmann, 1983). In brief, cancer cell lines (BT474, Hep-G2, SW620, Chago-K1 and KATO-III) and non-cancer cell lines were used and compared with five human cancer cell lines: human skin fibroblast ATCC no. CRL 1947 (CCD-986 SK), were seeded onto well plate at density of 5 × 10³ cell/well. Then, 2 μL of grape cell extract (0.1 g DW/mL) that had the maximum biomass, phenolics, resveratrol, and antioxidants (cells treated with 100 mg·L⁻¹ Na₂SeO₃, 50 mg·L⁻¹ Al₂(SO₄)₃, 5 μg·L⁻¹ chitosan, control 1 (NH₄NO₃), and control 2 (without treatments) was added to each well and incubated at 37 °C in a humidified atmosphere containing 5% CO₂ for 72 h. The compound doxorubicin was used as a positive control. MTT was added to cell supernatant at 10 μL/well. Then, the plates were wrapped with aluminum foil and incubated under the same condition for four additional hours. After removing untransformed MTT reagent, 100 μL of DMSO (dimethyl sulfoxide) was added to each well to dissolve MTT-formazan crystals, and absorbance was read at 570 nm using SpectraMax Plus384 microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA). The percentages of cytotoxicity and cell viability were calculated as follows and corrected for cytotoxicity due to DMSO in the control.

Cytotoxicity(%) = [(cells only – media)/ (cells only – treatment)] × 100

Viability(%) = 100 – % cytotoxicity

Statistical analysis. The randomized complete block design was used, with three replications, and each replicate was consisted with three experimental flask units. Data were collected in 7-d intervals at day 0, 7, 14, 21, and 28, and presented as mean ± sd (n = 3) of each independent treatment. Statistical analysis was performed using the SAS software for PC Version 9.1 (SAS Institute, Cary, NC). Significant differences between treatment means were separated by Duncan’s New Multiple Range Test at 95% confidence level.

Results and Discussion

Influence of chitosan and other chemical elicitor treatments on growth of V. vinifera cells. Growth of active cells is important for biochemically inducing health relevant bioactives in grape cell cultures. Overall, cell growth with NH₄NO₃ (control 1) was greater than that of with chitosan and other four chemical treatments regardless of sampling day intervals (Table 1). Cell dry weight of KH₂PO₄, KNO₃, Na₂SeO₃, and Al₂(SO₄)₃ treated cultured cells increased at 14 d and then decreased at 21 and 28 d. When compared with the four chemical elicitors, the biomass of grape cells treated with the chitosan elicitor was the lowest and it was equal to control 2. Among four chemicals and chitosan elicitors, KH₂PO₄ 200 mg·L⁻¹ resulted in the highest biomass (51.95 g·L⁻¹) at 14 d of culture, followed by Na₂SeO₃ 100 mg·L⁻¹ (45.0 g·L⁻¹). The finding that high concentrations of phosphate enhanced cell growth is in agreement with previous study on plant cell cultures, such as Nicotiana tabacum and Peganum harmala (Rao and Ravishankar, 2002). It is interesting to note that growth of cultured cells treated with Na₂SeO₃ 100 mg·L⁻¹ increased significantly because Se incorporation by grape cells could lead to the production of various seleno-containing compounds and seleno-amino acid derivatives, including Se-methyl-L-seleno-cysteine (MeSeCys) and glutamyl-methyl-seleno-cysteine. These seleno-amino acids and seleno-proteins might be useful for cell growth (Kapoor et al., 2012).

Moreover, results of this study showed that biomass of cells treated with high concentration of Na₂SeO₃ (100 mg·L⁻¹) was greater than cells treated with low concentration (50 mg·L⁻¹) at 7 and 14 d. Similarly, cell dry weight of grape cells increased with Al₂(SO₄)₃ 50 mg·L⁻¹ when compared with cells cultured with Al₂(SO₄)₃ 100 mg·L⁻¹. This response might be due to high concentrations of Al inhibited cell growth because of
the toxicity of excess Al in plant cells as previously reported in cucumber (Pereira et al., 2006) and pepper (Konarska, 2010). In KNO₃ treated culture, growth of grape cells remained unchanged during the growth period; however, the growth of the cells treated with of KNO₃ 500 mg L⁻¹ at 14 d (30.1 g L⁻¹) was higher than the control 2 (25.2 g L⁻¹).

In this study, 5 and 10 mg L⁻¹ chitosan as elicitor resulted in increased dry weight biomass with a 1.3- and 1.4-fold increase on day 21 when compared with the control 2. Limited information is available in the literature on the effect of chitosan on biomass/accumulation in grape cells. Chitosan was found to increase the biomass accumulation in V. vinifera cv. Gamay Freaux when treated with less than 25 mg L⁻¹ chitosan (Vuong et al., 2014), and stimulated the growth and yield of soybean seedlings (Lee et al., 2005) and sunflower sprouts (Cho et al., 2008). The biomass of cells treated with high concentration of chitosan (20 mg L⁻¹) was significantly lower than the cells treated with 10 mg L⁻¹ or lesser chitosan concentrations. High concentrations of chitosan (>10 mg L⁻¹) inhibited the growth of cultured cells by decreasing cell water potential (Nagella and Murthy, 2010).

**Influence of chitosan and other chemicals elicitors on phenolic content of V. vinifera cells.** In this study, chemical elicitors were targeted primarily to improve phenolic bioactive content in grape cell culture. Overall, the phenolic contents in cells treated with Na₂SeO₃ and Al₂(SO₄)₃ were greater compared with cells treated with KH₂PO₄, KNO₃, chitosan, control 1, and control 2 (Fig. 1). Phenolic contents in cells cultured with all elicitors were higher than that of control 2 at 7 d. However, when compared with control 1, only grape cells cultured with Al₂(SO₄)₃, Na₂SeO₃, and 5–10 mg L⁻¹ chitosan had higher phenolic content. Grape cells treated with 50 mg L⁻¹ Al₂(SO₄)₃ had the highest phenolic content at 7 d which was two and four times greater than control 1 and control 2, respectively.

The pattern of phenolic accumulation in grape cells in response to five elicitors changed from 14 to 21 d as the highest phenolics was observed in 100 mg L⁻¹ Na₂SeO₃ treated cells. Similarly at 14 d, phenolics increased significantly with elicitor treatments, with the exception of cells treated with KH₂PO₄. However, except chitosan treatments all other elicitor treatments resulted in reduction of phenolic content at 21 and 28 d. Therefore, the positive impact of chitosan for improving phenolic content of grape cell culture was more prominent in later growth stages. Grape cells treated with Na₂SeO₃ had the highest phenolic content at 21 and 28 d which was almost three times greater than the control 1 and control 2 (Fig. 1). Treatments of two elicitors, Na₂SeO₃ and Al₂(SO₄)₃, likely stressed the suspension cultured cells and subsequently induced higher phenolic accumulation (Karimi and Khatae, 2012).

Elicitor treatments with KH₂PO₄ and KNO₃ had less effect on the total phenolic content when compared with Na₂SeO₃, Al₂(SO₄)₃, and control 1, whereas cells cultured with KH₂PO₄ produced greater phenolics than KNO₃ treatment. Moreover, the greater phenolic accumulation was observed in the cells cultured with low concentrations of KH₂PO₄ 200 mg L⁻¹ and KNO₃ 500 mg L⁻¹ than higher concentrations (Fig. 1). Results were in agreement with previous study that found higher induction of phenolics (gymnemic acid) with KH₂PO₄ than KNO₃ treated cells of Gymnema sylvestre (Praveen et al., 2011) as.

The addition of chitosan as elicitor resulted in higher level of phenolic contents when compared with KH₂PO₄, KNO₃, and the control 2; however, it was less than that of cells treated with Na₂SeO₃ and Al₂(SO₄)₃ (Fig. 1). Chitosan and its derivatives exhibit elicitor activity by mimicking fungal cell wall interactions and subsequent induction of plant defense-related phytoalexins (Cho et al., 2008). Therefore, chitosan elicitor treatment could be a feasible way to trigger the biosynthesis of plant bioactive metabolites, including phenolics. The pattern of phenolic accumulation in the cells treated with chitosan was different from the other elicitors, as phenolic content of grape cell remained same during 28 d after chitosan elicitation treatment. These results suggested...
that production of plant bioactive compounds might be related to the chemical and elicitor-induced stress responses in the cultured cells (Vasconsuelo and Boland, 2007). In addition, Bourgaud et al. (1999) have demonstrated that chitosan could increase the accumulation of phenolics (coumestrol and daidzein isoflavone) in the tissue of Psoralea lachnostachys hairy root cultures. Moreover, the accumulation of phenolics in grape cells cultured with chitosan in this study might be related to its molecular size and associated functions, such as stress-induced aging and senescence relevant responses (Vasconsuelo and Boland, 2007).

**Influence of chitosan and other chemical elicitor treatments on resveratrol content of V. vinifera cells.** Resveratrol is an important health relevant phenolic bioactive compound commonly found in grape. Results indicated that KH2PO4 treatment was able to induce higher resveratrol content only at lower concentrations (200 mg L-1), especially at 14 d, (149.08 ± 10.99 mg g-1 DW) (Fig. 1). However, grape cells treated with KNO3 had no effect on resveratrol content, this might be due to having lower concentrations (200–2000 mg L-1) in this study, when compared with previous studies where they used higher concentrations, such as 3800 mg L-1 (Nagella and Murthy, 2010) and 5000 mg L-1 (Ali et al., 2006).

Resveratrol accumulation in grape cells treated with chemical elicitors, such as Na2SeO3 and Al2(SO4)3 resulted in significantly higher levels when compared with cells cultured with other macronutrient-based elicitor treatments, KH2PO4 and KNO3 (Fig. 1). Although cells treated with 100 mg L-1 Na2SeO3 resulted in greater resveratrol accumulation and it remained higher (174.50 ± 0.51 mg g-1 DW) during 7–28 d, cells treated with 50 mg L-1 Al2(SO4)3 had the highest resveratrol content (188.30 ± 0.98 mg g-1 DW). A relatively similar response was previously observed in pot-grown Assam tea where stress modulating Se and Al could influence the content of secondary metabolites, such as phenolic compounds (Sae-Lee et al., 2012). However, the phenolic biosynthesis induced by Se and Al has not been well documented, and it might be useful to determine if resveratrol contents would vary with respect to cis-resveratrol and other resveratrol derivatives (glycones).

Resveratrol contents of grape cells treated with chitosan elicitor were lower than that of Al2(SO4)3, Na2SeO3, and control 1 and 2 cells from 7 d to the end of the experiment (28 d). The amount of resveratrol in chitosan treated grape cell culture was ranged between 70.36 and 105.94 mg g-1 DW at 21 d, and 5 mg L-1 chitosan resulted in highest resveratrol content when compared with other doses of chitosan. Our findings are in agreement with the study of Bhuyan and Adachi (2003) where they reported that chitosan was less effective in inducing phenolic compounds than other biotic elicitors. By contrast, Korsangruang et al. (2010) have reported that an application of 10 mg L-1 chitosan induced highest isoflavonoids in P. candollii cultured cells at 21 d.

In this study, an increase in total phenolic and total resveratrol content was found in KH2PO4-treated grape cells. A positive correlation between total phenolic and resveratrol content was observed at 14–21 d (r² = 0.51–0.98, data not shown). Similarly, high and positive correlation (r² = 0.99) between phenolics and resveratrol content were found in KNO3-treated grape cells. However, in this study, phenolics and resveratrol content in KNO3 treated grape cells were less than previous study (Nagella and Murthy, 2010). It is possible that higher concentration of potassium is required to induce secondary metabolite transcripts and subsequent enhancement of phenolic antioxidants (Ali et al., 2006).

Furthermore, positive correlations between total phenolics and resveratrol contents were also observed in cultured grape cells treated with Na2SeO3, Al2(SO4)3, and chitosan at 14–21 d (r² = 0.44–0.26, 0.55–0.72, and 0.60–0.10, respectively). The Se has a catalytic role in a variety of enzymes that contain seleno-cysteine residues as a part of enhanced catalytic response for metabolic pathways related to biosynthesis of bioactive compounds (Kapoor et al., 2012), including phenolics and resveratrol. Therefore, higher concentrations of Se have potential to influence a greater response of secondary metabolite biosynthesis than lower concentrations (Arnault and Auger, 2006).

**Influence of chitosan and other chemical elicitor treatments on PAL activity of cultured grape cells.** Phenylalanine ammonia-lyase is one of the key enzymes in the metabolism of phenylpropanoids and secondary metabolites (Bauer et al., 2011). In this study, higher PAL enzyme activity was found with control 1 which was significantly higher than other chemical elicitor treatments (Fig. 2). This result suggested that increased phenolics and resveratrol content might suppress the PAL activity in grape cell culture during 28 d of growth. It is likely that PAL activity follows a cyclical mode of action with multiple isoforms responding to a state of metabolic flux and especially feed-back control as elicitor treatments resulted in higher phenolic and resveratrol. Therefore, it is reasonable to state that at higher levels phenolics potentially feed-back and inhibit the activity of PAL and this action may be reversed when phenolic content of cell reduces (Bauer et al., 2011).

**Influence of elicitor treatments on antioxidant activity of cultured grape cells: Based on DPPH free radical scavenging assay.** The DPPH free radical scavenging assay was used to determine the antioxidant activity of grape cell culture and to evaluate the effect of elicitor treatments on antioxidant potentials. Overall, antioxidant activity of grape cell culture increased at 7–14 d with elicitor and control treatments; however, it decreased at 21–28 d (Table 2). Grape cell culture treated with KH2PO4 and Al2(SO4)3 had higher DPPH radical scavenging activity when compared with other elicitor treatments. On the contrary, grape cell culture treated with 50 mg L-1 Na2SeO3 had lower antioxidant activities when compared with 100 mg L-1 Na2SeO3 treatment.

**Highest antioxidant activity was observed in grape cell culture treated with 50 mg L-1 Al2(SO4)3 at 14 d, with a DPPH scavenging capacity of 186.6 mg TE/100 g DW (Table 2).** Although the free radical scavenging activity (based on DPPH) of suspended cultured cells decreased when Al2(SO4)3 content in the medium was 100 mg L-1 when compared with lower concentration (50 mg L-1). However, KH2PO4 resulted in higher antioxidant activity when compared with other elicitor treatments and control. Secondary metabolites, such as phenolic compounds in particular are involved in plant responses to abiotic stresses and provide a significant contribution to the overall antioxidant activity of plant cells/tissues/organs (Giri et al., 2012; He and Yan, 2013; Korsangruang et al., 2010). It is known that environmental stress, including excess or starvation of plant nutrients, may increase the production of phenolic compounds and associated antioxidant activity (Kovacick and Backor, 2007). In this study, 50–100 g L-1 Al2(SO4)3, 100 mg L-1 Na2SeO3 at 7–14 d, and 200–1000 mg L-1 KH2PO4 at 14 d increased the phenolic and resveratrol contents and higher phenolics of treated grape cell culture may also have contributed to their high antioxidant activity based on DPPH radical scavenging assay.

**Influence of chitosan and other chemical elicitor treatments on antioxidant activity of cultured grape cells: Based on ABTS** + radical scavenging activity. Antioxidant activity of cultured grape cells treated with 200 mg L-1 KH2PO4 was highest at 7 d, whereas 500 mg L-1 KH2PO4 elicitor treatments resulted in highest antioxidant activity at 14 d with values of 207.0 and 210.0 mm TE/100 g DW, respectively (Table 2). These results were in contrast with observation of phenolics and resveratrol contents which were highest in grape cells treated with 100 mg L-1 Na2SeO3 and 50 mg L-1 Al2(SO4)3. It is hypothesized that phenolics and resveratrol could also be efficient scavengers of free radicals (He and Yan, 2013). However, differences in antioxidant activities measured by different assays are likely due to the mechanism of action of the DPPH or ABTS + radical scavenging assays with higher response of ABTS toward water soluble fractions. In this study, the ABTS + method appeared to be more sensitive in determining antioxidant activity of bioactive compounds, such as phenolics, especially resveratrol. Because ABTS could determine the antioxidant capacity at low inhibitory concentrations, the radical-scavenging capacity detected by ABTS method was greater than that with DPPH. The finding of this study was similar to the result of suspension cultures of Habe-naria edgeworthii callus reported by Giri et al. (2012). Moreover, abiotic elicitors
could upregulate the gene expression of the defense related protein (PR protein) (Belhadj et al., 2008), as well as the PAL (the key enzymes in the phenylpropanoid pathway via stilbene and chalcone synthase) to produce phenolic compounds (Bauer et al., 2011). The PR protein then could stimulate endogenous trans-resveratrol accumulation, as well as can mobilize their release to the extracellular medium. In addition, oxidative dimerization could occur from resveratrol to e-viniferin or δ-viniferin (Jeandet et al., 2002) during growth of cell in suspension medium.

Influence of the chitosan and chemicals of elicited grape cells on inhibition of cancer cell lines. Overall, results indicated that grape cell culture extracts could inhibit BT474 breast cancer (18% to 68% inhibition), Chago-K1 lung cancer, Hep-G2 liver cancer, KATO-III stomach cancer, and SW620 colon cancer cells (Table 3). Extracts of elicitor-treated grape cell culture had significant cytotoxicity against all cancer cell lines when compared with the control 1 and control 2. When five dilutions (1:10, 1:20, 1:40, 1:80, and 1:160) of extracts of previously chemical/elicitor-treated grape cells were evaluated, a significant dose-dependent response on the viability of the cancer cells lines was observed (data not shown). The 1:10 diluted grape cell culture extracts treated with Na2SeO3 100 mg·L⁻¹ significantly reduced viability of cancer cell lines when compared with the control 1 and control 2. The maximum inhibitory effect on SW620 cells was observed with the Na2SeO3 100 mg·L⁻¹ treated grape cells and after 1:10 dilution. Overall, elicitor treatment such as 100 mg·L⁻¹ Na2SeO3 resulted in a high percentage reduction of viability of cancer cells. More specifically, extracts of Na2SeO3-treated grape cells inhibited the breast cancer cell line (BT474) (37%), lung cancer cell (Chago-K1) (46%), liver cancer cell (Hep-G2) (65%), stomach cancer cell (KATO-III) (67%), and colon cancer cell (SW620) (68%). The extracts of Na2SeO3-treated grape cell culture likely had enhanced levels of seleno-amino acids and seleno-proteins (Kapoor et al., 2012) which might synergistically contribute to the inhibition of cancer cell lines along with higher level of phenolic metabolites. The role of seleno-metabolites have potential relevance in overall cancer prevention against liver, stomach, and colon cancer, as well as lung cancer and leukemia, as reported by Arnault and Auger (2006). This chemopreventive properties could be even more effective when combined with enhanced phenolic phytochemicals, such as resveratrol in grape cell cultures. Moreover, the inhibition of KATO-III and SW620 cells was greater than 50% with grape cell extracts that were previously treated with Na2SeO3 100 mg·L⁻¹, Al2(SO4)3 50 mg·L⁻¹, and chitosan 5 mg·L⁻¹. In this study, the extracts of previously treated cultured grape cells (control 1, control 2, Na2SeO3 100 mg·L⁻¹, Al2(SO4)3 and chitosan 5 mg·L⁻¹) did not inhibit the growth of normal cells (noncancer) skin fibroblast, which indicated that elicitor-treated grape cell culture are safe against normal cells. The improvement of phenolic content and especially of resveratrol might have significant relevance for enhancing cytotoxicity against cancer cells and can be targeted for overall and wider health benefits linked to cancer and other NCDs (Ting et al., 2015).

Conclusions

Chitosan and other chemical elicitors were targeted to improve phenolic bioactive content and associated antioxidant and anti-cancer properties in grape cell culture. Overall, higher phenolic content and associated antioxidant activity was found in elicitor treated grape cell culture. Grape cells treated with 100 mg·L⁻¹ Na2SeO3 and with 50 mg·L⁻¹ Al2(SO4)3 resulted in higher production of phenolics and resveratrol compared with cells treated with KNO3 and chitosan. Higher antioxidant activity (DPPH based) was also observed in grape cell cultures treated with KH2PO4, Al2(SO4)3, and Na2SeO3, and it was positively correlated with...
Table 2. The effect of potassium dihydrogen phosphate (KH$_2$PO$_4$), potassium nitrate (KNO$_3$), sodium selenite (Na$_2$SeO$_3$), aluminium sulphate (Al$_2$(SO$_4$)$_3$), and chitosan on antioxidant activity of Vitis vinifera cv. Pok Dum cell cultures at 7, 14, 21, and 28 d.

| Treatment          | Conc. | Day 7 | Day 14 | Day 21 | Day 28 |
|--------------------|-------|-------|--------|--------|--------|
|                    |       | DPPH (mM TE/100g DW) |        | ABTS (mM TE/100g DW) |        |
|                    | 200 mg·L$^{-1}$ | 80.0 ± 1.18 cB | 114.8 ± 5.78 cdA | 83.0 ± 3.62 aB | 59.0 ± 1.62 aC |
|                    | 500 mg·L$^{-1}$ | 77.0 ± 2.90 cB | 108.3 ± 6.09 deA | 60.0 ± 2.96 cC | 51.0 ± 1.83 bD |
|                    | 1,000 mg·L$^{-1}$ | 72.0 ± 3.42 dB | 107.6 ± 7.24 cA | 58.0 ± 2.43 cC | 43.0 ± 2.53 cD |
| KH$_2$PO$_4$      |       |        |        |        |        |
| KNO$_3$           | 500 mg·L$^{-1}$ | 53.0 ± 3.19 gb | 24.0 ± 0.72 fA | 14.0 ± 0.29 hiD | 66.0 ± 2.12 bD |
|                   | 1,000 mg·L$^{-1}$ | 50.0 ± 1.01 gb | 21.0 ± 1.73 hc | 17.0 ± 1.40 gbD | 76.8 ± 2.87 hcD |
|                   | 2,000 mg·L$^{-1}$ | 45.0 ± 3.53 bB | 26.0 ± 3.38 gb | 14.0 ± 0.80 hiD | 75.0 ± 1.79 aC |
| Na$_2$SeO$_3$     | 50 mg·L$^{-1}$ | 62.2 ± 1.06 fB | 122.9 ± 2.24 cA | 22.3 ± 1.57 hc | 182.0 ± 2.55 bC |
|                   | 100 mg·L$^{-1}$ | 120.1 ± 2.00 bB | 162.5 ± 1.40 bA | 38.8 ± 1.74 efC | 117.8 ± 7.09 fB |
|                   | 200 mg·L$^{-1}$ | 123.1 ± 2.51 bB | 165.1 ± 9.70 bA | 49.3 ± 1.22 dc | 117.8 ± 7.09 fB |
| Al$_2$(SO$_4$)$_3$ | 50 mg·L$^{-1}$ | 182.1 ± 9.13 aA | 186.6 ± 2.10 aA | 70.0 ± 7.10 bB | 117.8 ± 7.09 fB |
|                   | 100 mg·L$^{-1}$ | 123.1 ± 2.51 bB | 165.1 ± 9.70 bA | 49.3 ± 1.22 dc | 117.8 ± 7.09 fB |
|                   | 200 mg·L$^{-1}$ | 34.5 ± 4.02 iA | 26.4 ± 1.31 gB | 23.6 ± 1.63 gbB | 117.8 ± 7.09 fB |
| Chitosan          | 10 mg·L$^{-1}$ | 35.1 ± 1.81 fA | 35.5 ± 3.41 gA | 35.2 ± 2.39 fA | 138.0 ± 5.94 dA |
|                   | 20 mg·L$^{-1}$ | 25.4 ± 1.45 jB | 34.1 ± 2.95 gA | 28.3 ± 3.60 gB | 138.0 ± 5.94 dA |
| Control 1 (NH$_4$NO$_3$) | 68.9 ± 3.06 eB | 108.7 ± 14.59 eA | 41.6 ± 0.16 eC | 26.1 ± 1.28 eB | 138.0 ± 5.94 dA |
| Control 2 (without elicitor) | 60.6 ± 1.05 fB | 100.2 ± 9.09 dA | 26.0 ± 3.10 cA | 22.3 ± 1.76 eC | 138.0 ± 5.94 dA |

**F-test**
**CV (%)**
**LSD**

NH$_4$NO$_3$ = ammonium nitrate; DPPH = 1, 1-diphenyl-2-picrylhydrazyl; ABTS = 2,2-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); TE = Trolox equivalents; DW = dry weight.

Means of three replicates ±SD in the same column (a, b, c, d,...) or in the same line (A–D) with different letters are significantly (P ≤ 0.05), separated by Duncan’s New Multiple Range Test.

**Significantly different at P ≤ 0.01.

Table 3. Effect of elicitor-treated grape cell culture extracts on inhibitory activity (%) against five human cancer cell lines.

| Elicited cultured grape cells | Normal cells | BT474 (breast cancer) | Chago-K1 (lung cancer) | Hep-G2 (liver cancer) | KATO-III (stomach cancer) | SW620 (colon cancer) |
|------------------------------|--------------|-----------------------|------------------------|-----------------------|--------------------------|----------------------|
| Control 1 (NH$_4$NO$_3$)     | NC           | 18 ± 5.94 c            | 27 ± 4.28 c            | 27 ± 6.70 d           | 56 ± 2.66 c              | 42 ± 2.50 c          |
| Control 2 (without elicitor) | NC           | 26 ± 5.56 b            | 31 ± 3.36 bc           | 37 ± 6.40 bc          | 60 ± 2.80 b              | 55 ± 4.16 b          |
| Na$_2$SeO$_3$ 100 mg·L$^{-1}$| NC           | 37 ± 2.03 a            | 46 ± 3.45 a            | 65 ± 3.08 a           | 67 ± 1.64 a              | 68 ± 2.27 a          |
| Al$_2$(SO$_4$)$_3$ 50 mg·L$^{-1}$ | NC          | 26 ± 3.74 b            | 36 ± 0.48 b            | 43 ± 6.32 b           | 64 ± 2.17 a              | 47 ± 3.84 c          |
| Chitosan 5 mg·L$^{-1}$       | NC           | 33 ± 6.00 ab           | 18 ± 3.64 d            | 31 ± 3.12 cd          | 58 ± 1.02 bc             | 57 ± 4.92 b          |

**F-test**
**CV (%)**
**LSD**

NC = noncytotoxic; NH$_4$NO$_3$ = ammonium nitrate; Na$_2$SeO$_3$ = sodium selenite; Al$_2$(SO$_4$)$_3$ = aluminium sulphate.

Means of three replicates ±SD. Means within a column followed by the same letters are not significantly different, P > 0.05.

**Significantly different at P ≤ 0.01.

**Table 1.** The effect of potassium dihydrogen phosphate (KH$_2$PO$_4$), potassium nitrate (KNO$_3$), sodium selenite (Na$_2$SeO$_3$), aluminium sulphate (Al$_2$(SO$_4$)$_3$), and chitosan on antioxidant activity of Vitis vinifera cv. Pok Dum cell cultures at 7, 14, 21, and 28 d.

**Table 2.** The effect of elicitor-treated grape cell culture extracts on inhibitory activity (%) against five human cancer cell lines.

**Table 3.** The effect of elicitor-treated grape cell culture extracts on inhibitory activity (%) against five human cancer cell lines.
phenolic and resveratrol content in the same grape cell culture. The extracts of grape cells treated with 100 mg L⁻¹ Na₂SeO₃ also resulted in the highest cytotoxicity against cancer cell lines. Therefore, these elicitor treatments in select doses can be safely targeted to improve phenolic bioactive content and associated antioxidant and anticancer properties of cell culture and which can be further confirmed with future in vivo and animal model studies. This study provides biochemical foundation for wider application of elicitation strategy for inducing bioactives in plant cell cultures for chemoprevention and to counter oxidative stresses commonly associated with cancer and other NCDs.

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