



# Differential expression of anthocyanin biosynthesis genes in *Daucus carota* callus culture in response to ammonium and potassium nitrate ratio in the culture medium

Kirti R. Saad<sup>1</sup> · Gyanendra Kumar<sup>1</sup> · Parvatam Giridhar<sup>1</sup> · Nandini Prasad Shetty<sup>1</sup>

Received: 22 May 2018 / Accepted: 24 September 2018 / Published online: 29 September 2018  
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

## Abstract

Anthocyanins are major water-soluble and dynamic colouring plant pigment present in plant tissues with the high antioxidant properties. The role of ammonium and potassium nitrate in the culture medium on anthocyanin augmentation is probed thoroughly, but the mechanism of its biosynthesis continues to be unclear. Hence, the present study was undertaken to optimise nitrate ratio in the culture medium for anthocyanin augmentation and examination of its biosynthesis pathway in callus culture of *Daucus carota*. MS basal medium fortified with various ratio of  $\text{NH}_4\text{NO}_3$ : $\text{KNO}_3$  was employed to find their impact on biomass, anthocyanin augmentation and the expression profile of anthocyanin biosynthesis genes in the callus culture. The data indicated that the highest anthocyanin content ( $9.30 \pm 0.25$  mg/100 g FW) was seen in callus grown on the medium supplemented with 20.0 mM  $\text{NH}_4\text{NO}_3$ :37.6 mM  $\text{KNO}_3$  and the least was seen in the medium which contained 40.0 mM  $\text{NH}_4\text{NO}_3$ :18.8 mM  $\text{KNO}_3$  ( $2.74 \pm 0.27$  mg/100 g FW). This indicates an optimal concentration of  $\text{NH}_4\text{NO}_3$ : $\text{KNO}_3$  ratio is essential to produce a higher amount of anthocyanin in in vitro culture. Meanwhile, anthocyanin biosynthesis genes were differentially expressed as confirmed by qRT-PCR in the time interval of 5, 10, 15, 20 and 25 days. The transcript levels of nine anthocyanin biosynthesis genes were increased in the response of varying  $\text{NH}_4\text{NO}_3$ : $\text{KNO}_3$  ratio in the medium. The transcript level of early genes PAL, 4CL, CHS and CHI increased by 19.5, 21.0, 16.2 and 9.98-fold, respectively, compared with control. In addition, late biosynthesis genes LDOX and UFGT resulted in the transcript level of 11.3 and 13.6-fold, respectively.

**Keywords** Cyanidin 3-glucoside · Gene expression · Nitrate ratio · qRT-PCR · 4-Coumaroyl:CoA-ligase

## Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
4CL	4-Coumaroyl:CoA-ligase
C4H	Cinnamate 4-hydroxylase
CHI	Chalcone isomerase
CHS	Chalcone synthase
DFR	Dihydroflavonol reductase
F3H	Flavanone 3-hydroxylase
FW	Fresh weight
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
Kin	Kinetin

LDOX	Leucoanthocyanidin dioxygenase
MS	Murashige and Skoog
PAL	Phenylalanine ammonia lyase
qRT-PCR	Quantitative real-time-polymerase chain reaction
UFGT	UDP glucose:flavonoid-3-O-glucosyltransferase

## Introduction

Plant secondary metabolites have many applications in pharmaceuticals and food industries as a food additive, drugs, flavours, fragrances, dyes, pigments, pesticides and cosmetic industries (Hussain et al. 2012). Anthocyanin, a water-soluble pigment, belongs to flavonoid family of polyphenol phytochemicals. It provides bright red–orange to blue–violet colour to many fruits and vegetables. Anthocyanin present in the form of aglycone attached to different

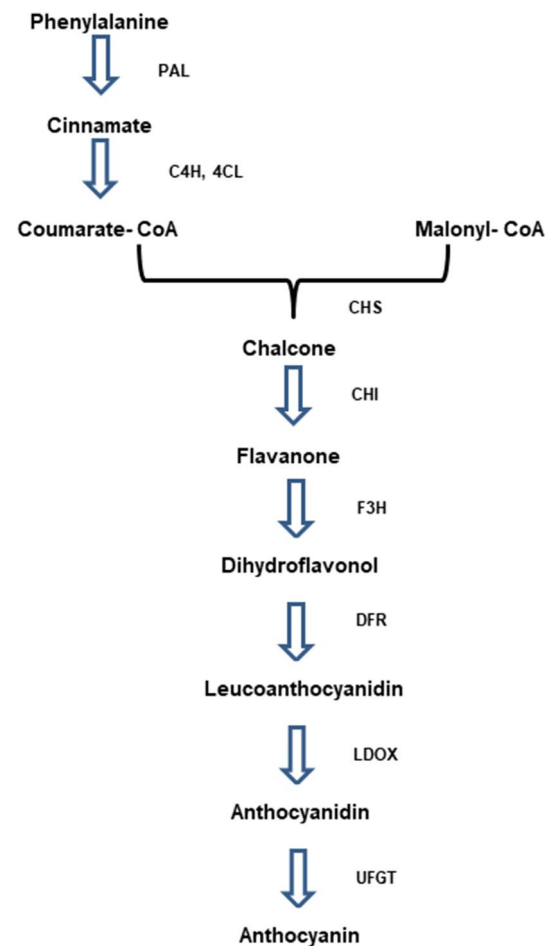
✉ Nandini Prasad Shetty  
nandinips@cftri.res.in

<sup>1</sup> Plant Cell Biotechnology Department, CSIR-Central Food Technological Research Institute, Mysore, Karnataka 570 020, India

sugar moiety in the plants. Important anthocyanin includes cyanidin, delphinidin, peonidin, pelargonidin, petunidin, malvidin and it is stored in vacuoles (Zhang et al. 2002). Anthocyanin plays important role in plants viz. pollination, reproduction and protect against UV light (Samanta et al. 2011). Intake of anthocyanin is reported to be beneficial in the treatment of diabetic retinopathy (Nabavi et al. 2015), prevention of non-alcoholic fatty liver (Valenti et al. 2013), protection from DNA damage (Acquaviva et al. 2003), anti-inflammatory activity, lipid peroxidation (Ramirez-Tortosa et al. 2001), inhibit tumour formation (Kang et al. 2003) and has strong antioxidant properties (Madrigal-Santillan et al. 2014).

Anthocyanin accumulation in callus cultures is known to be induced by many factors such as MS medium constituents, growth conditions, environmental conditions, elicitation and precursor feeding (Ali et al. 2017). In addition, medium constituents such as MS salt strength, sucrose (Hiratsuka et al. 2001), nitrogen (Irshad et al. 2018), phosphate (Saad et al. 2018) and plant growth hormone (Jamwal et al. 2018) in the culture medium is known to be influence the anthocyanin accumulation. In vitro produced anthocyanin exhibits better stability than that of the anthocyanin from in vivo extracts (Madhavi et al. 1996). Therefore, optimization of the medium constituents is crucial for improving the commercial production of anthocyanin. MS medium constitutes with inorganic metal compounds which plays the important role in growth, development of plant cells and also involves an in vitro production of secondary metabolites. Ammonium and potassium nitrate is one of the inorganic compounds which play a significant role in plant metabolic process for the production of anthocyanin and other secondary metabolites (Shehata et al. 2014). Zahedzadeh et al. (2015) reported that changing the ratio of ammonium to potassium nitrate into the MS medium resulted in a significantly higher accumulation of anthocyanin in apple.

Anthocyanins are synthesised from the phenylpropanoid pathway illustrated in Fig. 1. It has been studied in many plant species such as *Arabidopsis thaliana* (Solfanelli et al. 2006), *Vitis vinifera* (Hiratsuka et al. 2001), *Pyrus communis* (Li et al. 2012), *Solanum tuberosum* (Lu et al. 2006), *Malus sieversii* (Wan et al. 2015) and *Rosa hybrid* (Hennayake et al. 2006). Enzymes involved in the biosynthesis pathways are divided into two classes—early genes which include—phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumaroyl:CoA-ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′5′-hydroxylase (F3′H) and late genes—dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS), also called leucoanthocyanidin dioxygenase (LDOX) and UDP glucose:flavonoid-3-*O*-glucosyltransferase (UFGT). Many studies have been carried out to show the importance of



**Fig. 1** Phenylpropanoid pathway for the biosynthesis of anthocyanin. Enzymes include: *PAL* phenylalanine ammonia lyase, *C4H* cinnamate 4-hydroxylase, *4CL* 4-coumaroyl: CoA-ligase, *CHS* chalcone synthase, *CHI* chalcone isomerase, *F3H* flavanone 3-hydroxylase, *DFR* dihydroflavonol reductase, *ANS* anthocyanidin synthase, *LDOX* leucoanthocyanidin dioxygenase, *UFGT* UDP glucose:flavonoid-3-*O*-glucosyltransferase

nitrate in anthocyanin accumulation. However, their effect on early and late anthocyanin biosynthetic gene in in vitro has not been discussed. Molecular understanding of nitrate response will be useful in anthocyanin improvement and also enhance their production. An early study with maize seedling demonstrated higher expression of *PAL*, *CHS*, *4CL* and *CHI* genes when treated with low temperature (Christie et al. 1994). Studies have shown that late anthocyanin biosynthesis genes (*DFR*, *LDOX* and *UFGT*) regulated by jasmonate in *Arabidopsis* (Shan et al. 2009). Abscisic acid (ABA) and gibberellic acid (GA) had shown a synergistic effect on the expression of *DFR* genes in *Arabidopsis* (Loreti et al. 2008). However, there are no conclusive reports on the effects of  $\text{NH}_4\text{NO}_3:\text{KNO}_3$  ratio on the expression of the anthocyanin biosynthesis pathway genes in *Daucus carota* callus culture.

The focus of the present study is to examine the importance of ammonium and potassium nitrate ratio on anthocyanin augmentation and expression profile of nine anthocyanin biosynthesis genes in callus culture of *D. carota*. The expression levels of nine genes encoding key enzymes (PAL, 4CL, C4H, CHS, CHI, F3H, DFR, LDOX and UFGT) for anthocyanin biosynthesis were analysed by qRT-PCR. The correlation between the augmentation of anthocyanin and expression on its biosynthesis genes were discussed to shed the light in the molecular level.

## Materials and methods

### Plant materials and culture conditions

*Daucus carota* seeds (Atomic red var.) were collected from plantfro.dk, Denmark. Surface sterilization of seeds was done using 4% (v/v) sodium hypochlorite (Hi-media, Mumbai) for 10 min followed by washing (4–5 times) with autoclaved distilled water. Sterilized seeds were inoculated in the MS basal media (Murashige and Skoog 1962) containing 3% (w/v) sucrose (Hi-media, Mumbai) and 0.5% (w/v) cleriGar (Hi-media, Mumbai) at  $25 \pm 2$  °C in the dark. One-month-old seedlings were used as explant for callus induction.

### Callus induction

Seedlings obtained were used as explants for callus induction. Leaf, node and internode part of seedlings were chopped and cultured in MS basal medium containing 9.1  $\mu\text{M}$  2,4-dichlorophenoxyacetic acid (2,4-D, Hi-media, Mumbai), 2.32  $\mu\text{M}$  Kinetin (Kin, Hi-media, Mumbai), 3% (w/v) sucrose, and 0.5% (w/v) cleriGar. Medium pH was adjusted to 5.8 and autoclaved for 15 min at 121 °C. Callus cultures were maintained in dark condition at  $25 \pm 2$  °C. Callus was sub-cultured every 4 weeks of interval in the same medium for 2 months.

### Anthocyanin induction

Green and friable callus obtained was inoculated in medium supplemented with 11.41  $\mu\text{M}$  indole-3-acetic acid (IAA) and 0.93  $\mu\text{M}$  Kinetin (Kin) for anthocyanin induction. Both these growth hormones were procured from Hi-media, Mumbai. Callus cultures were grown under the culture condition of 16/8 h (light/dark) of lighting with white fluorescent tubes ( $28 \mu\text{Mol s}^{-1} \text{m}^{-2}$ ) at  $25 \pm 2$  °C. Callus cultures were maintained in the same medium and growth condition. Sub-cultured were done for 6 months in every 4 weeks to get uniform pigmentation.

### $\text{NH}_4\text{NO}_3$ and $\text{KNO}_3$

MS basal medium contains full strength of  $\text{NH}_4\text{NO}_3$  (20.0 mM, Hi-media, Mumbai) and  $\text{KNO}_3$  (18.8 mM, Hi-media, Mumbai) as the two macro-elements. In this study, the MS basal concentration of these two nitrogen sources in the medium was replaced by different combinations to investigate their impact on anthocyanin content and anthocyanin biosynthetic profile. The combinations are represented in Table 1. Callus used in this experiment has been maintained for 6 months. Callus of 0.2 g fresh weight (FW) was inoculated in the medium with different combinations of  $\text{NH}_4\text{NO}_3$  and  $\text{KNO}_3$  ratio. Callus was harvested in time interval of 5, 10, 15, 20 and 25 days to measure anthocyanin content and isolation of RNA.

### Anthocyanin analysis

Total anthocyanin content was analysed by the pH-differential method of Wrolstad et al. (2005). Pigmented callus was extracted using acidic methanol (0.1% v/v HCL) and kept for shaking overnight in dark. Crude extract (0.5 ml) were added separately to 0.5 ml potassium chloride buffer (0.025 M at pH 1.0) and 0.5 ml sodium acetate buffer (0.4 M at pH 4.5). The absorbance of the extract was recorded at 520 nm and 700 nm using a UV-Vis spectrophotometer (UV 1800 Shimadzu, Japan). Total anthocyanin content was calculated based on a cyanidin 3-glucoside standard (Sigma Aldrich, Bangalore). The absorbance ( $A$ ) of the diluted sample was then calculated as follows:  $A = (A_{520 \text{ nM}} - A_{700 \text{ nM}}) \text{ pH } 1.0 - (A_{520 \text{ nM}} - A_{700 \text{ nM}}) \text{ pH } 4.5$ :

$$\text{TAC} = [(A \times \text{MW} \times \text{DF} \times \text{VE}) / \epsilon \times l \times \text{FW}] \times 1000,$$

where TAC = total anthocyanin content,  $A$  is the difference of the absorbance, MW = molecular weight (449.2  $\text{g mol}^{-1}$  for cyanidin 3-glucoside), DF = dilution factor, VE = volume of extract,  $\epsilon$  = extinction coefficient (26,900  $\text{l mol}^{-1} \text{cm}^{-1}$  for cyanidin 3-glucoside), FW = fresh weigh of callus (g).

**Table 1** Callus culturing medium composition with different ratio of ammonium and potassium nitrate

Medium	Macro-elements		Biomass (g)
	$\text{NH}_4\text{NO}_3$ (mM)	$\text{KNO}_3$ (mM)	
1	20.0	0	1.62 $\pm$ 0.11
2	20.0	9.4	1.91 $\pm$ 0.08
3	20.0	37.6	1.24 $\pm$ 0.14
4	0	18.8	2.05 $\pm$ 0.12
5	10.0	18.8	1.71 $\pm$ 0.06
6	40.0	18.8	0.98 $\pm$ 0.02
Control	20.0	18.8	1.60 $\pm$ 0.03

The results were reported as mg equivalents of cyanidin 3-glucoside per 100 g of fresh weight (mg cya3G/100 g FW). The above result values are means of three replicate from same experiment.

### High performance liquid chromatography (HPLC) analysis

Anthocyanin analysis was carried out in callus culture using HPLC. Known quantity of callus mass was harvested on day 20 and extracted for anthocyanin with acidic methanol (0.1% v/v HCL). The extracts were homogenised and kept for shaking overnight in dark. A crude extract of anthocyanin was partially purified using Amberlite XAD-7 column with the method of Saad et al. (2018). Partially purified sample (20 µl) was injected into the Shimadzu LC 10-AD HPLC equipped with a dual pump and a UV detector (Model SPD-M20A, Shimadzu, Japan). A Column used for separation of the anthocyanin was poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm (Agilent Technologies, USA). The mobile phase was consisting of solvent A (1% formic acid in water) and solvent B (1% formic acid in acetonitrile). The flow rate was 1 ml/min and wavelength of the UV detector was set at 520 nm. The solvent gradient was 0–1 min, 5% B; 1–3 min, 5–8% B; 3–6 min, 8–15% B; and 6–14 min, 15% B; and 14–15 min, 15–5%.

### RNA isolation and cDNA synthesis

Callus was grown in control and modified media containing 20.0 mM NH<sub>4</sub>NO<sub>3</sub>:37.6 mM KNO<sub>3</sub> ratio was harvested in time interval of 5, 10, 15, 20 and 25 days to isolate total RNA using the cetyl-trimethylammonium bromide (CTAB) method (Salmona et al. 2008). RNA quality were analysed by running the RNA sample in 1.2% formaldehyde agarose gel electrophoresis and concentration of total RNA was analysed by Nanodrop spectrophotometer (Thermo scientific, Bangalore). The purity of RNA was determined by absorbance ratio (A260/A280 and A260/A230) and RNA integrity was evaluated by comparing the intensity of the 28S/18S rRNA ratio. DNA contamination was removed by the RNA sample using DNase I enzyme treatment (Thermo scientific, Bangalore). 1 µg of total RNA was reverse transcribed using Verso cDNA Synthesis Kit (Thermo scientific, Bangalore) in a final volume of 20 µl according to manufacturer's instructions.

### Quantitative real-time PCR analysis

All the anthocyanin biosynthesis genes were analysed by real-time PCR (Quant studio 5, Applied Biosystem, India) using SYBR Green Real-Time PCR Master Mix (Takara, Bangalore). Real-time PCR was conducted in a 10 µl reaction volume containing 1 µl diluted cDNA, 5 µM forward and reverse primer (Table 2) and 5 µl sybr green. The

**Table 2** Sequences of forward and reverse primers used to amplify genes involved in anthocyanin biosynthesis of *Daucus carota*

Gene name	GenBank accession number	Primer sequence	Product length (bp)
Phenylalanine ammonia lyase (PAL)	MG931011	Forward primer-ATTTGGAGGAGGTGAAGAGGATG Reverse primer-GCGAGAGATGATAGTGGCGTC	119
Cinnamate 4-hydroxylase (C4H)	MG931005	Forward primer-GTGGAGGCCAACGAAATG Reverse primer-CGTCCGATTGTGATACCGAGA	107
4-coumaroyl:CoA-ligase (4CL)	MG931016	Forward primer-AGAGCCAAGTTTCCTAATGCCA Reverse primer-TCCCGTTGATTCCTTGGTAG	198
Chalcone synthase (CHS)	MG931012	Forward primer-TGAAGCCTGAGAACTGAAG Reverse primer-AGAACACCCCAATCAAGTC	154
Chalcone isomerase (CHI)	MG931010	Forward primer-TCCTCCAGGTACACCAATG Reverse primer-CTCCTTCCGTTACCGAAATTC	163
Flavanone 3-hydroxylase (F3H)	MG931006	Forward primer-GATGAGCGTCCTAAGGTTG Reverse primer-AAAATCCCCCAGTCTTCAC	137
Dihydroflavonol reductase (DFR)	MG931009	Forward primer-CGGTGTACGATGAATCCAAC Reverse primer-ATGCTGCCTTCTCTGCTAATG	105
Leucoanthocyanidin dioxygenase (LDOX)	MG931013	Forward primer-GGCACTACCTTAGAATCTCC Reverse primer-CCTTCATCACTCTTTTCCTCC	139
UDP glucose flavonoid 3-O-glucosyltransferase (UFGT)	MG931014	Forward primer-GCAATCCTATCCCATCCTTC Reverse primer-CAACCCCGACTCTAACACC	175
Actin (ACT)	MG931007	Forward primer-TATGAGCAAGAGTTGGAGACT Reverse primer-CATAGATGGCTGGAAGAGGAC	136

qRT-PCR reaction was performed with three technical and three biological replicates. The PCR reaction performed was as follows: initial denaturation at 95 °C for 30 s followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 58 °C for 30 s. Expression of actin was used to serve as a control. For comparing the gene expression levels under control and modified medium were expressed as the fold changes and statistical analyses were also performed to show the significant difference. The relative expression ratio of each gene was calculated using  $2^{-\Delta\Delta C_T}$  (Livak and Schmittgen 2001).

### Statistical analyses

Values expressed in this study represent mean  $\pm$  SD of triplicates from the same experiment. All the experiments were performed twice. The statistical significance of differences was determined by one-way ANOVA analysis for gene expression and two-way ANOVA (IBM SPSS 23 Statistics, Armonk) for biomass and total anthocyanin content followed by a Tukey's multiple range tests at the significance level of  $p < 0.05$ .

## Results

### Callus and anthocyanin induction

Seeds were germinated in 7 days after the inoculation in the MS medium. Seedlings obtained were used as explant for callus induction. Callus was induced from the wounded area of the leaf, node and internode on MS basal medium supplemented by 2,4-D (9.1  $\mu$ M) and Kin (2.32  $\mu$ M) after 4 weeks of inoculation. Green and friable callus were used to induce anthocyanin pigmentation by sub-culturing into the medium comprising IAA (11.41  $\mu$ M) and Kin (0.93  $\mu$ M) with 3% sucrose. Six sub-cultures at 4-week interval led to the initiation of pigmentation from callus. Coloured callus was separated and regularly inoculated into the IAA and Kin containing medium for uniform pigmentation.

### Anthocyanin augmentation in response to $\text{NH}_4\text{NO}_3$ : $\text{KNO}_3$

Modification of ammonium and potassium nitrate ratio in the medium strongly influenced the growth of callus and augmentation of anthocyanin in callus culture of *D. carota* (Fig. 2). MS basal medium comprises of ammonium nitrate (20.0 mM) and potassium nitrate (18.8 mM) as macro inorganic nitrogen compounds. Maximum biomass was obtained in the medium with 0:18.8 (2.05  $\pm$  0.12 g) followed by 20.0:9.4 (1.91  $\pm$  0.08 g) and 10.0:18.8 (1.71  $\pm$  0.06 g) mM  $\text{NH}_4\text{NO}_3$  to  $\text{KNO}_3$  ratio. The fresh weight of callus grown in the medium with

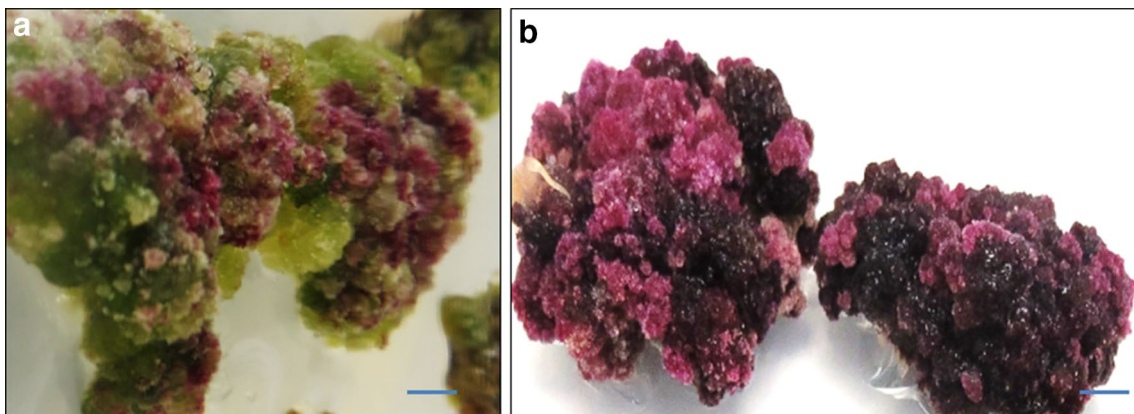
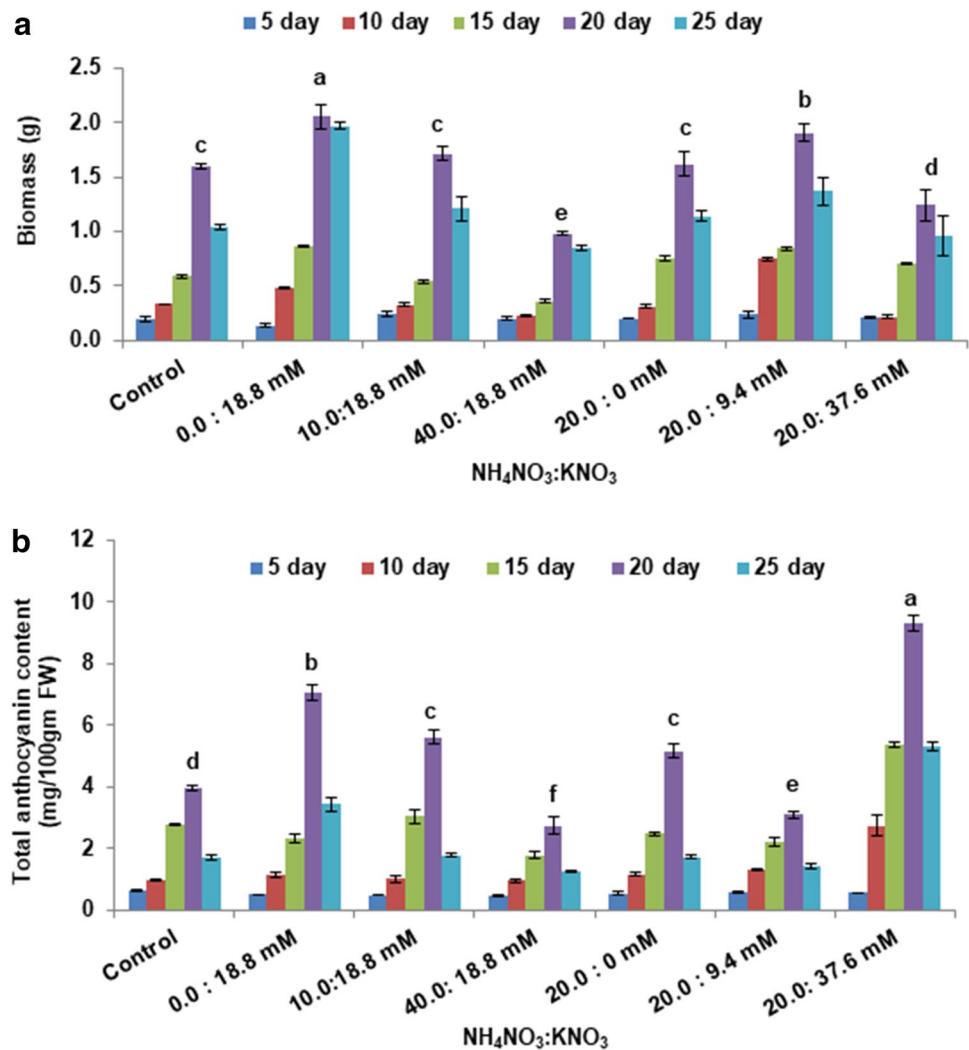
20.0 mM  $\text{NH}_4\text{NO}_3$ :37.6 mM  $\text{KNO}_3$  (1.24  $\pm$  0.14 g) showed reduced biomass compare to control (1.60  $\pm$  0.03 g). In addition, we observed growth of cells was inhibited by 40.0 mM  $\text{NH}_4\text{NO}_3$ :18.8 mM  $\text{KNO}_3$  (0.98  $\pm$  0.02 g) ratio than control. This finding indicated that the presence of ammonium nitrate concentration in the MS medium shows an adverse effect on cell growth, whereas increasing in potassium nitrate slightly decreased the growth of callus compare to control (Fig. 2a).

Anthocyanin quantification was conducted in the callus obtained from medium containing different ratio of  $\text{NH}_4\text{NO}_3$  to  $\text{KNO}_3$  after every 5 days of interval (Fig. 2b). Anthocyanin augmentation was started from day 5th until 20th, after which there was a decline in accumulation by 25th day because of nutrient depletion and toxin accumulation into the medium. Highest anthocyanin content (9.30  $\pm$  0.25 mg/100 g FW) was observed in the medium containing 20.0:37.6 mM ratio of  $\text{NH}_4\text{NO}_3$  to  $\text{KNO}_3$  at day 20. Medium with deficient  $\text{NH}_4\text{NO}_3$  and basal level of  $\text{KNO}_3$  (18.8 mM) enhanced anthocyanin by 78.48%, whereas increasing the concentration of  $\text{NH}_4\text{NO}_3$  from 0 to 10.0 mM (half strength) slightly increases the anthocyanin content by 42.09%. However, doubling the concentration of  $\text{NH}_4\text{NO}_3$  (40.0 mM) into the medium decreased the anthocyanin content by 30.77%. Callus treated with 20.0:9.4 and 20.0:37.6 mM  $\text{NH}_4\text{NO}_3$ : $\text{KNO}_3$  ratio increased anthocyanin augmentation by 99.52% and 135.13%, respectively. This study finds that increasing the concentration of  $\text{KNO}_3$  from basal level (18.8 mM) to two-fold strength (37.6 mM) in MS medium gives the significant increase in anthocyanin accumulation, whereas other combination did not show any significance. Enhancement of anthocyanin content in the control and modified medium with the ratio of  $\text{NH}_4\text{NO}_3$ : $\text{KNO}_3$  shows in Fig. 3. Furthermore, the results suggest that the presence of  $\text{KNO}_3$  in the medium is crucial for anthocyanin augmentation in callus culture of *D. carota*.

### High performance liquid chromatography (HPLC) analysis

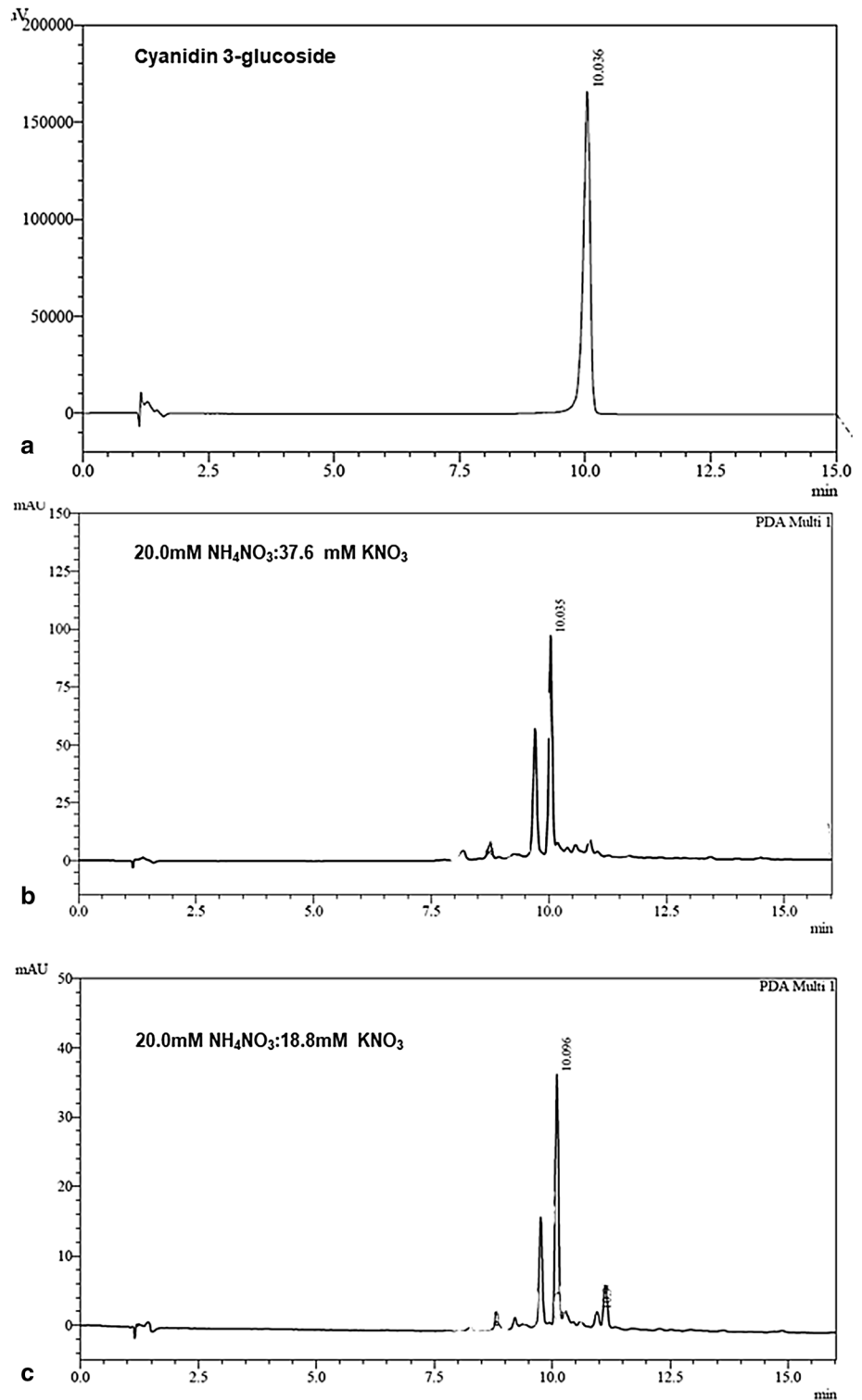
Anthocyanin extract from control and treated callus was partially purified using Amberlite XAD-7 column and analysed using HPLC (Fig. 4). The anthocyanin composition of carrot (Atomic red var.) consists mainly of cyanidin. The major peak of cyanidin 3-glucoside is shown in Fig. 4a. Callus has grown in control (20.0 mM  $\text{NH}_4\text{NO}_3$ :18.8 mM  $\text{KNO}_3$ ) and modified media (20.0 mM  $\text{NH}_4\text{NO}_3$ :37.6 mM  $\text{KNO}_3$ ), whereas it showed the major peak at retention time of 10 min as compared with the standard (cyanidin 3-glucoside). The major peak in control and modified medium grown callus represented area of 34% and 41% of the total area at 520 nm, respectively. An increasing level of  $\text{KNO}_3$

**Fig. 2** Influence of different ratio of ammonium nitrate and potassium nitrate on **a** biomass and **b** total anthocyanin content in callus culture of *D. carota*. The growth of callus and total anthocyanin content was analysed in 5 days of time interval for 25 days under 16/8 h photoperiod. Results were analysed by two-way ANOVA, using Tukey's test. The values were the means of three replicates with standard deviations. Values sharing a same letter are not significantly different at  $p < 0.05$



**Fig. 3** **a** Callus culture of *D. carota* grown on control medium (20.0 mM NH<sub>4</sub>NO<sub>3</sub>:18.8 mM KNO<sub>3</sub>) and **b** callus grown on the modified medium supplemented with 20.0 mM NH<sub>4</sub>NO<sub>3</sub>:37.6 mM KNO<sub>3</sub> at day 20 (bar=5 cm)

**Fig. 4** Chromatographic profile of anthocyanin extract at 520 nm. Effect of  $\text{NH}_4\text{NO}_3$  to  $\text{KNO}_3$  ratio on anthocyanin production. Callus were inoculated in control (20.0 mM  $\text{NH}_4\text{NO}_3$ :18.8 mM  $\text{KNO}_3$ ) and modified media with 20.0 mM  $\text{NH}_4\text{NO}_3$ :37.6 mM  $\text{KNO}_3$  for 20 days. Anthocyanin was partially purified and quantify by HPLC. **a** Cyanidin 3-glucoside, **b** media with 20.0 mM:37.6 mM ( $\text{NH}_4\text{NO}_3$ : $\text{KNO}_3$ ) and **c** control



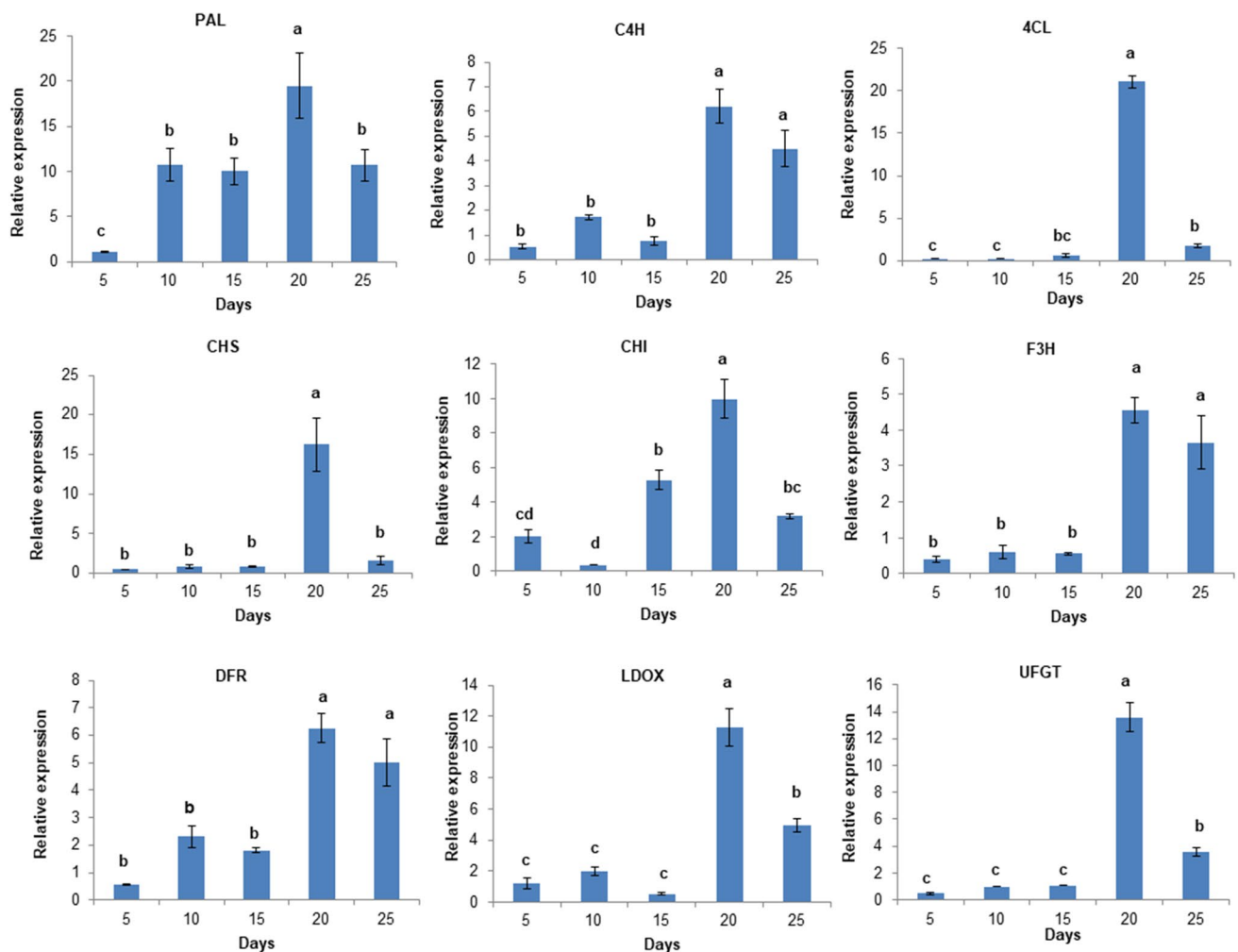
from basal level increased the peak height, peak area and values of the cyanidin 3-glucoside compare to control (Fig. 4b, c).

#### $\text{NH}_4\text{NO}_3$ : $\text{KNO}_3$ ratio regulates the expression of anthocyanin biosynthetic genes

The expression studies of nine anthocyanin biosynthesis genes were carried out to understand the regulation of anthocyanin biosynthesis genes in the response of ammonium and

potassium nitrate ratio incorporated into the medium. The nine genes studied were PAL, 4CL, C4H, CHS, CHI, F3H, DFR, LDOX and UFGT. In the present study, we found that media supplemented with 20.0 mM  $\text{NH}_4\text{NO}_3$  and 37.6 mM  $\text{KNO}_3$  showed significantly higher accumulation of anthocyanin in callus culture of *D. carota* in comparison to control and other combinations. Media with 20.0 mM  $\text{NH}_4\text{NO}_3$  and 37.6 mM  $\text{KNO}_3$  ratio was thus selected to understand the transcript level of anthocyanin biosynthesis genes in response to control (20.0 mM  $\text{NH}_4\text{NO}_3$  and 18.8 mM  $\text{KNO}_3$ ). The estimation of gene expression level was carried out based on CT value obtained from cDNA extracted from control and modified medium growing callus in an interval of 5, 10, 15, 20 and 25 days after inoculation. To understand which biosynthesis genes will play key role in increasing anthocyanin content in response to ammonium to potassium nitrate ratio in the medium was analysed by

qRT-PCR (Fig. 5). The transcript levels of all the genes involved in anthocyanin biosynthesis were increased by the modified concentration of 20.0 mM  $\text{NH}_4\text{NO}_3$  to 37.6 mM  $\text{KNO}_3$  ratio in the medium. The transcript levels of all the biosynthetic pathway genes were higher on day 20. In the study, the expression of the early anthocyanin biosynthesis genes PAL, 4CL and CHS was increased by 19.5, 21.0 and 16.2-fold, whereas *C4H* and *F3H* showed slightly increased by 6.2 and 4.5-fold compared to control, respectively. In addition, late biosynthesis genes (*DFR*, *LDOX* and *UFGT*) were also studied in the modified medium (Fig. 5), where *LDOX* and *UFGT* transcripts were greatly increased, by 13.6 and 11.3-fold, whereas *DFR* showed moderately increased by 6.3-fold. Thus the expression of all the nine anthocyanin biosynthesis genes in response to the addition of ammonium and potassium nitrate ratio in the medium were presented significant increases in anthocyanin accumulation.



**Fig. 5** Relative expression profile of anthocyanin biosynthetic genes in callus culture of *D. carota*. Real-time PCR was used to analyze the expression levels of *PAL*, *C4H*, *4CL*, *CHS*, *CHI*, *F3H*, *DFR*, *LDOX* and *UFGT*. All real-time PCR reactions were normalized using the Ct

value corresponding to control. Different letters above the bars indicate significantly different values ( $p < 0.05$ ) calculated using one-way ANOVA followed by a Tukey's multiple range tests



## Discussion

To establish callus culture and induction of secondary metabolites, different media composition is required to promote a shift from growth state to metabolite inducing stage. In the present study, medium supplemented with 2,4-D and Kin were responsible for callus induction, whereas IAA and Kin were responsible for the anthocyanin accumulation in the callus culture of *D. carota*. In callus culture of *M. malabathricum* high anthocyanin content occurred with 0.25 mg L<sup>-1</sup> BAP and 0.5 mg L<sup>-1</sup> NAA into the medium (Chan et al. 2010), whereas the highest anthocyanin production stimulated at a combination of 8 μM BAP and 2 μM NAA in callus culture of *Oxalis linearis* (Meyer and Van Staden 1995). Combination of 2 mg L<sup>-1</sup> BAP and 1 mg L<sup>-1</sup> NAA gave high anthocyanin production in callus culture of *Crataegus sinaica* (Maharik et al. 2009). Hence, different genotype responds differently to different media composition and plant hormone.

In this study, callus was treated with different ratio of ammonium and potassium nitrate for anthocyanin augmentation. The growth of the callus was consistent with the basal (20 mM) and reduced (9.4 mM) level of NH<sub>4</sub>NO<sub>3</sub> into the MS medium, whereas with increased level (40 mM) of NH<sub>4</sub>NO<sub>3</sub> the growth was affected adversely. The highest biomass was seen in the medium without ammonium nitrate. The reason behind this might be presence of higher concentration of ammonium which can easily diffuse and accumulate in the tissue and become toxic if not consumed. In the presence of low concentration of ammonium into the medium, cells consumed it effortlessly for metabolism.

Strategies for enhancing the anthocyanin production in the callus culture require the optimization of medium constituents for stable and continuous production. Nitrogen is one of the vital macro elements in the basal MS medium and plays important role in increasing the production of anthocyanin. Different ratio of NH<sub>4</sub>NO<sub>3</sub> and KNO<sub>3</sub> in the medium affects the growth of cells as well as the production of secondary metabolites. The optimum concentration of NH<sub>4</sub>NO<sub>3</sub> to KNO<sub>3</sub> ratio was 20.0 and 37.6 mM in the medium to produce the maximum amount of anthocyanin compared to control and other ratios. Medium with double strength of KNO<sub>3</sub> (37.6 mM) increased the anthocyanin production, while doubling the concentration of NH<sub>4</sub>NO<sub>3</sub> (40.0 mM) in the medium failed to enhance the anthocyanin content, since higher ammonium concentration in the medium increases the acidity of the medium which results in less consumption of potassium which directly influence the production of anthocyanin (Al-Ibresam and Al-Meer 2008). These results indicate that the increased ratio of KNO<sub>3</sub> with the basal level of NH<sub>4</sub>NO<sub>3</sub> in the medium had a positive effect on enhancement of anthocyanin content.

Similar result was observed in the enhancement of rutin production in callus and root culture of *Morus alba* which efficiently stimulates by NO<sub>3</sub><sup>-</sup> compare to NH<sub>4</sub><sup>+</sup> in the medium (Lee et al. 2011). In addition, eliminating NH<sub>4</sub>NO<sub>3</sub> from the culture media enhanced the growth and tannin production in callus cultures of *Quercus acutissima* (Tanaka et al. 1995). The outcome of the present study revealed that KNO<sub>3</sub> is an important nitrogen source compare to NH<sub>4</sub>NO<sub>3</sub> in the MS medium. Increasing the KNO<sub>3</sub> concentration in the MS medium leads to the enhancement of anthocyanin production in callus culture of *D. carota*. This phenomenon also reported in an enhancement of saponin content in hairy roots of *Talinum paniculatum* in the presence of double strength of potassium nitrate into the MS media (Manuhara et al. 2015). According to Hirasuna et al. (1991) findings, the presence of nitrate-sensitive tonoplast ATPase was responsible for the augmentation of anthocyanin in grape culture. The presence of nitrogen source (ammonium and potassium nitrate) in the medium performs oxidation reduction enzyme reaction which produces ATP responsible for cell division and induction of the phenolic compound (Al-Turki et al. 2010).

Anthocyanin in callus culture of *D. carota* was determined by the analytical method like spectroscopy and chromatography. The qualification and quantification of anthocyanin from callus culture involve the extraction using methanolic HCL buffer and partially purified with Amberlite XAD-7 column. HPLC method quantified cyanidin 3-glucoside as the major anthocyanin present in callus culture. Abe et al. (2008) also reported the presence of cyanidin 3-glucoside in suspension culture of *D. carota* by HPLC method.

To find the effect of nitrates on the regulation and expression of the early genes in the anthocyanin biosynthesis pathway viz, PAL, 4CL and CHS in particular shows a relationship with the degree of anthocyanin augmentation in callus culture of *D. carota*. However, the expression of late anthocyanin biosynthesis pathway genes LDOX and UFGT significantly positively correlated with the anthocyanin augmentation. Shi and Xie (2010) have reported the effect of ammonium nitrate and potassium nitrate level on anthocyanins pathway gene. The medium was supplemented with three different combinations of NH<sub>4</sub>NO<sub>3</sub>:KNO<sub>3</sub> ratio viz., 20.0/18.8 mM; 10.0/9.4 mM and 0/9.4 mM. Medium with 10.0 NH<sub>4</sub>NO<sub>3</sub>:9.4 mM KNO<sub>3</sub> showed higher transcript level of *PAP1*, *CHS*, *DFR*, and *LDOX* in rosette leaves of pap1-D of *Arabidopsis thaliana*. Wang et al. (2016) reported the increased expression of *ANS*, *F3H*, *CHS*, *DFR*, and *UFGT* genes in callus cultures of *Malus sieversii* under light condition than in darkness while declining expression levels in response to increase in temperature. Expression of *CHI* was higher with low temperature, whereas the expression of *CHS* and *F3H* was inhibited by high temperature. Work done in *Chrysanthemum morifolium* for flavonoid metabolism in

leaves found an increased level of flavonoid and *PAL* activity in response to nitrogen deficiency (Liu et al. 2010). In *Solanum lycopersicum* leaves, *PAL*, *CHS* and *F3H* expression increased due to nitrogen depletion (Lovdal et al. 2010). Similarly, in *Arabidopsis*, low concentration of nitrogen resulted in up-regulation of *PAL*, *CHS*, *F3H*, *DFR* and *DOX* genes (Peng et al. 2008).

## Conclusion

The present study reports the influence of ammonium and potassium nitrate ratio on anthocyanin augmentation and its biosynthesis genes. Callus inoculate into the medium containing 20.0 mM  $\text{NH}_4\text{NO}_3$  and 37.6 mM  $\text{KNO}_3$  lead to maximum augmentation of anthocyanin. Major anthocyanin was identified from partially purified XAD-7 extracts of callus culture of *D. carota* and represented as glucoside of cyanidin by HPLC method. Result showed the significant effect of medium optimization on the augmentation of anthocyanin in control and modified medium grown callus. Expression profile of all the nine genes of anthocyanin biosynthesis showed correlation with anthocyanin content. The results suggest that incorporation of ammonium nitrate with potassium nitrate (double strength) into the medium shows a synergistic effect on enhancement of anthocyanin augmentation. An optimised medium constituent gives the background for the commercial production of secondary metabolites to replace the synthetic colour. The increased use of genetic tools and regulation of pathways for secondary metabolism will provide the basis for the production of commercially acceptable level of products. Understanding the role of nitrates or inorganic metals ions on anthocyanin biosynthesis pathways helps to improve the larger scale production.

**Acknowledgements** This research was supported by the Central Food Technological Research Institute (CFTRI). We thank to our Director CSIR-CFTRI who provided insight and expertise that greatly assisted the research. We would also like to thank Council of Scientific and Industrial Research (CSIR) for fellowship.

## Compliance with ethical standards

**Conflict of interest** We declare that we have no conflict of interest.

## References

- Abe Y, Sawada A, Momose T, Sasaki N, Kawahara N, Kamakura H, Ozeki Y (2008) Structure of an anthocyanin–anthocyanin dimer molecule in anthocyanin-producing cells of a carrot suspension culture. *Tetrahedron Lett* 49(51):7330–7333. <https://doi.org/10.1016/j.tetlet.2008.10.041>
- Acquaviva R, Russo A, Galvano F, Galvano G, Barcellona ML, Volti GL, Vanella A (2003) Cyanidin and cyanidin 3-*O*- $\beta$ -D-glucoside as DNA cleavage protectors and antioxidants. *Cell Biol Toxicol* 19(4):243–252. <https://doi.org/10.1023/B:CBTO.0000003974.27349.4e>
- Ali M, Abbasi BH, Ahmad N, Khan H, Ali GS (2017) Strategies to enhance biologically active-secondary metabolites in cell cultures of *Artemisia*—current trends. *Crit Rev Biotechnol* 37(7):833–851. <https://doi.org/10.1080/07388551.2016.1261082>
- Al-Ibresam OT, Al-Meer UN (2008) Effect of ammonium and potassium nitrate in some characteristics of callus and somatic embryos of date palm (*Phoenix dactylifera* L.) by *in vitro*. *Basrah J Date Palm Res* 7:1–16. <https://doi.org/10.3923/biotech.2014.116.125>
- Al-Turki S, Shahba MA, Stushnoff C (2010) Diversity of antioxidant properties and phenolic content of date palm (*Phoenix dactylifera* L.) fruits as affected by cultivar and location. *J Food Agric Environ* 8:253–260. <https://doi.org/10.1234/4.2010.1495>
- Chan LK, Koay SS, Boey PL, Bhatt A (2010) Effects of abiotic stress on biomass and anthocyanin production in cell cultures of *Melastoma malabathricum*. *Biol Res* 43(1):127–135. <https://doi.org/10.4067/S0716-97602010000100014>
- Christie PJ, Alfenito MR, Walbot V (1994) Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta* 194(4):541–549. <https://doi.org/10.1007/BF00714468>
- Hennayake CK, Takagi S, Nishimura K, Kanechi M, Uno Y, Inagaki N (2006) Differential expression of anthocyanin biosynthesis genes in suspension culture cells of *Rosa hybrida* cv. Charleston. *Plant Biotechnol* 23(4):379–385. <https://doi.org/10.5511/plantbiotech.23.379>
- Hirasuna TJ, Shuler ML, Lackney VK, Spanswick RM (1991) Enhanced anthocyanin production in grape cell cultures. *Plant Sci* 78(1):107–120. [https://doi.org/10.1016/0168-9452\(91\)90167-7](https://doi.org/10.1016/0168-9452(91)90167-7)
- Hiratsuka S, Onodera H, Kawai Y, Kubo T, Itoh H, Wada R (2001) ABA and sugar effects on anthocyanin formation in grape berry cultured *in vitro*. *Sci Hortic* 90(1–2):121–130. [https://doi.org/10.1016/S0304-4238\(00\)00264-8](https://doi.org/10.1016/S0304-4238(00)00264-8)
- Hussain MS, Fareed S, Saba Ansari M, Rahman A, Ahmad IZ, Saeed M (2012) Current approaches toward production of secondary plant metabolites. *J Pharm Bioallied Sci* 4(1):10. <https://doi.org/10.4103/0975-7406.92725>
- Irshad M, Debnath B, Mitra S, Arafat Y, Li M, Sun Y, Qiu D (2018) Augmentation of anthocyanin in callus cultures of red-pod okra [*Abelmoschus esculentus* (L.) Hongjiao] in response to light and nitrogen levels. *Plant Cell Tissue Organ Cult* 134:29–39. <https://doi.org/10.1007/s11240-018-1397-6>
- Jamwal K, Bhattacharya S, Puri S (2018) Plant growth regulator mediated consequences of secondary metabolites in medicinal plants. *J Appl Res Med Aromat Plants*. <https://doi.org/10.1016/j.jarmp.2017.12.003>
- Kang SY, Seeram NP, Nair MG, Bourquin LD (2003) Tart cherry anthocyanins inhibit tumour development in ApcMin mice and reduce proliferation of human colon cancer cells. *Cancer Lett* 194(1):13–19. [https://doi.org/10.1016/S0304-3940\(02\)00583-9](https://doi.org/10.1016/S0304-3940(02)00583-9)
- Lee Y, Lee DE, Lee HS, Kim SK, Lee WS, Kim SH, Kim MW (2011) Influence of auxins, cytokinins, and nitrogen on production of rutin from callus and adventitious roots of the white mulberry tree (*Morus alba* L.). *Plant Cell Tissue Organ Cult* 105(1):9–19. <https://doi.org/10.1007/s11240-010-9832-3>
- Li L, Ban ZJ, Li XH, Wu MY, Wang AL, Jiang YQ, Jiang YH (2012) Differential expression of anthocyanin biosynthetic genes and transcription factor PcMYB10 in pears (*Pyrus communis* L.). *PLoS One* 7(9):e46070. <https://doi.org/10.1371/journal.pone.0046070>
- Liu W, Zhu DW, Liu DH, Geng MJ, Zhou WB, Mi WJ, Hamilton D (2010) Influence of nitrogen on the primary and secondary

- metabolism and synthesis of flavonoids in *Chrysanthemum morifolium* Ramat. *J Plant Nutr* 33(2):240–254. <https://doi.org/10.1080/01904160903434287>
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the  $2^{-\Delta\Delta C_T}$  method. *Methods* 25:402–408. <https://doi.org/10.1006/meth.2001.1262>
- Loreti E, Povero G, Novi G, Solfanelli C, Alpi A, Perata P (2008) Gibberellins, jasmonate and abscisic acid modulate the sucrose-induced expression of anthocyanin biosynthetic genes in *Arabidopsis*. *New Phytol* 179(4):1004–1016. <https://doi.org/10.1111/j.1469-8137.2008.02511.x>
- Lovdal T, Olsen KM, Slimestad R, Verheul M, Lillo C (2010) Synergistic effects of nitrogen depletion, temperature, and light on the content of phenolic compounds and gene expression in leaves of tomato. *Phytochemistry* 71(5–6):605–613. <https://doi.org/10.1016/j.phytochem.2009.12.014>
- Lu Q, Yang Q, Zou H (2006) Effects of cerium on augmentation of anthocyanins and expression of anthocyanin biosynthetic genes in potato cell tissue cultures. *J Rare Earth* 24(4):479–484. [https://doi.org/10.1016/S1002-0721\(06\)60147-6](https://doi.org/10.1016/S1002-0721(06)60147-6)
- Madhavi DL, Juthangkoon S, Lewen K, Berber-Jimenez MD, Smith MAL (1996) Characterization of anthocyanins from *Ajuga pyramidalis* Metallica Crispa cell cultures. *J Agric Food Chem* 44(4):1170–1176. <https://doi.org/10.1021/jf950504u>
- Madrigal-Santillan E, Madrigal-Bujaidar E, Alvarez-Gonzalez I, Sumaya-Martinez MT, Gutierrez-Salinas J, Bautista M, Morales-Gonzalez JA (2014) Review of natural products with hepatoprotective effects. *World J Gastroenterol* 20(40):14787. <https://doi.org/10.3748/wjg.v20.i40.14787>
- Maharik N, Elgengaihi S, Taha H (2009) Anthocyanin production in callus cultures of *Crataegus sinica* Boiss. *Int J Acad Res* 1(1):30–34. <https://doi.org/10.7813/2075-4124.2009/1-1/A.3>
- Manuhara YSW, Kristanti AN, Utami ESW, Yachya A (2015) Effect of sucrose and potassium nitrate on biomass and saponin content of *Talinum paniculatum* Gaertn. hairy root in balloon-type bubble bioreactor. *Asian Pac J Trop Biomed* 5(12):1027–1032. <https://doi.org/10.1016/j.apjtb.2015.09.009>
- Meyer HJ, Van Staden J (1995) The *in vitro* production of an anthocyanin from callus cultures of *Oxalis linearis*. *Plant Cell Tissue Organ Cult* 40(1):55–58. <https://doi.org/10.1007/BF00041119>
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15(3):473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- Nabavi SF, Habtemariam S, Daglia M, Shafiqhi N, Barber AJ, Nabavi SM (2015) Anthocyanins as a potential therapy for diabetic retinopathy. *Curr Med Chem* 22(1):51–58. <https://doi.org/10.2174/0929867321666140815123852>
- Peng M, Hudson D, Schofield A, Tsao R, Yang R, Gu H, Rothstein SJ (2008) Adaptation of *Arabidopsis* to nitrogen limitation involves induction of anthocyanin synthesis which is controlled by the *NLA* gene. *J Exp Bot* 59(11):2933–2944. <https://doi.org/10.1093/jxb/ern148>
- Ramirez-Tortosa C, Andersen OM, Gardner PT, Morrice PC, Wood SG, Duthie SJ, Duthie GG (2001) Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats. *Free Radic Biol Med* 31(9):1033–1037. [https://doi.org/10.1016/S0891-5849\(01\)00618-9](https://doi.org/10.1016/S0891-5849(01)00618-9)
- Saad KR, Parvatam G, Shetty NP (2018) Medium composition potentially regulates the anthocyanin production from suspension culture of *Daucus carota*. *3 Biotech* 8(3):134. <https://doi.org/10.1007/s13205-018-1146-x>
- Salmona J, Dussert S, Descroix F, De Kochko A, Bertrand B, Joet T (2008) Deciphering transcriptional networks that govern *Coffea arabica* seed development using combined cDNA array and real-time RT-PCR approaches. *Plant Mol Biol* 66(1–2):105–124. <https://doi.org/10.1007/s11103-007-9256-6>
- Samanta A, Das G, Das SK (2011) Roles of flavonoids in plants. *Int J Pharm Sci Technol* 6(1):12–35
- Shan X, Zhang Y, Peng W, Wang Z, Xie D (2009) Molecular mechanism for jasmonate-induction of anthocyanin augmentation in *Arabidopsis*. *J Exp Bot* 60(13):3849–3860. <https://doi.org/10.1093/jxb/erp223>
- Shehata WF, Aldaej MI, Alturki SM, Ghazzawy HS (2014) Effect of ammonium nitrate on antioxidants production of date palm (*Phoenix dactylifera* L.) *in vitro*. *Biotechnology* 13(3):116–125. <https://doi.org/10.3923/biotech.2014.116.125>
- Shi MZ, Xie DY (2010) Features of anthocyanin biosynthesis in pap1-D and wild-type *Arabidopsis thaliana* plants grown in different light intensity and culture media conditions. *Planta* 231(6):1385–1400. <https://doi.org/10.1007/s00425-010-1142-9>
- Solfanelli C, Poggi A, Loreti E, Alpi A, Perata P (2006) Sucrose-specific induction of the anthocyanin biosynthetic pathway in *Arabidopsis*. *Plant Physiol* 140(2):637–646. <https://doi.org/10.1104/pp.105.066688>
- Tanaka N, Shimomura K, Ishimaru K (1995) Tannin production in callus cultures of *Quercus acutissima*. *Phytochemistry* 40(4):1151–1154. [https://doi.org/10.1016/0031-9422\(95\)00378-K](https://doi.org/10.1016/0031-9422(95)00378-K)
- Valenti L, Riso P, Mazzocchi A, Porrini M, Fargion S, Agostoni C (2013) Dietary anthocyanins as nutritional therapy for non-alcoholic fatty liver disease. *Oxid Med Cell Longev*. <https://doi.org/10.1155/2013/145421>
- Wan H, Zhang J, Song T, Tian J, Yao Y (2015) Promotion of flavonoid biosynthesis in leaves and calli of ornamental crabapple (*Malus* sp.) by high carbon to nitrogen ratios. *Front Plant Sci* 6:673. <https://doi.org/10.3389/fpls.2015.00673>
- Wang N, Zhang Z, Jiang S, Xu H, Wang Y, Feng S, Chen X (2016) Synergistic effects of light and temperature on anthocyanin biosynthesis in callus cultures of red-fleshed apple (*Malus sieversii* f. *niedzwetzkyana*). *Plant Cell Tissue Organ Cult* 127(1):217–227. <https://doi.org/10.1007/s11240-016-1044-z>
- Wrolstad RE, Durst RW, Lee J (2005) Tracking color and pigment changes in anthocyanin products. *Trends Food Sci Technol* 16:423–428. <https://doi.org/10.1016/j.tifs.2005.03.019>
- Zahedzadeh F, Kakavand F, Mahna N (2015) Effects of carbohydrate, light, nitrogen and magnesium on *in vitro* production of anthocyanin in apple. *Int J Biosci* 6(5):250–260. <https://doi.org/10.12692/ijb/6.5.250-5>
- Zhang W, Curtin C, Franco C (2002) Towards manipulation of post-biosynthetic events in secondary metabolism of plant cell cultures. *Enzym Microb Technol* 30(6):688–696. [https://doi.org/10.1016/S0141-0229\(02\)00041-8](https://doi.org/10.1016/S0141-0229(02)00041-8)