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Effect of mevalonic acid feeding on the terpenoid pathway in *Catharanthus roseus* cell suspension cultures

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Abstract

The terpenoid pathway is considered as a limiting factor in the biosynthesis of terpenoid indole alkaloid (TIA) in *Catharanthus roseus* cell suspension cultures. The limitation could be due to the competition for the isopentenyl diphosphate::dimethylallyl diphosphate utilized for different terpenoid pathways in the plastids, and includes outflow of plastidial MEP pathway precursors to other compartments and associated terpenoid pathways, both of which would result in precursor shortage to the iridoid-TIA pathway. In this study, the upstream precursor of the mevalonate pathway, i.e. mevalonic acid was fed to the *C. roseus* cell suspension cultures to evaluate the changes of metabolic flows into the different terpenoid pathways with specific attention to the distribution of C5-units into sterols (triterpenoid, C30), carotenoids (tetraterpenoid, C40), and TIAs (monoterpenoid, C10). This study showed that the sterol level was increased 72 hours after feeding mevalonic acid at a low level (0.2 mM final concentration). However, the carotenoid level was not different from the control and TIA level did not show a clear enhancement. When a higher level (3.3 mM final concentration) of mevalonic acid was fed into the cell cultures, the loganic acid level was higher than the control after 24 hours, but the TIA level was not significantly different. After 72 hours, loganic acid and TIA levels were significantly decreased and tryptamine had accumulated in the mevalonate fed cells. While carotenoids remained unaffected, sterols level was 41% increased after 24 hours and the accumulation was 111% higher than the control level after 72 hours. These results indicate that feeding mevalonic acid does not increase the levels of TIA and carotenoid in the MEP pathway but increased fluxes in the mevalonate pathway leading to sterols.

Introduction

In *Catharanthus roseus*, the terpenoid indole alkaloids (TIAs), of which some are pharmaceutically important compounds, derive from the metabolic precursors of two different biosynthetic pathways, i.e. tryptamine from the shikimate-tryptophan pathway and secologanin from the terpenoid-iridoid pathway (El-Sayed and Verpoorte 2007). Terpenoid biosynthesis in plants occurs via two distinct metabolic routes, the mevalonate (MVA) pathway which is localized in the cytosol, and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway which is present in the plastids (Rohmer 1999). Both MVA and MEP pathway produce the terpenoid building blocks: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), but each pathway leads to a distinct set of terpenoid derivatives (**Fig. 1**).

The iridoids in *C. roseus* are synthesized from geranyl diphosphate (GPP) supplied by the MEP pathway (Contin et al. 1998). Precursor feeding studies in *C. roseus* cell suspension cultures showed that the terpenoid pathway leading to the iridoids is the rate-limiting step for TIA biosynthesis (Moreno et al. 1993; Whitmer et al. 2002). This could be due to the lack in the biosynthesis of precursors or intermediates associated to low gene expression or enzyme activity in the MEP pathway. In addition, it might be due to the competition at the level of IPP::DMAPP and GPP with conversion steps favoring either geraniol and derived monoterpenoids and iridoids, versus geranylgeranyl diphosphate (GGPP) and derived carotenoids (Saiman et al. 2014).

There is evidence of interaction between the IPP::DMAPP pools in the cytosolic mevalonate and plastidial MEP pathways (Arigoni et al. 1997; Schuhr et al. 2003; Hemmerlin et al. 2003; Laule et al. 2003). Laule et al. (2003) showed an existence of cross-talk or interaction between the cytosolic mevalonate and the plastidial MEP pathway by studying the levels of gene transcriptions and several metabolites (sterols, carotenoids, chlorophylls) after adding specific inhibitors of the respective pathways in *Arabidopsis thaliana* seedlings. Their results show that sharing of isopentenyl precursors between both pathways may occur but only in a unidirectional process, from plastid to cytosol, i.e. the MEP pathway may feed intermediates to the cytosolic mevalonate pathway and not vice versa. This redirection of carbon resources could indicate that IPP::DMAPP from the MEP pathway are leaked to the cytosolic terpenoid pathway, when there is an increased demand but a limiting supply of the IPP::DMAPP C5-units from the mevalonate pathway. Under such circumstances, the cytosolic terpenoid pathways could be competing for the same intermediates as the carotenoid and the iridoid-TIA pathway, which could result in limitations of precursor supply to the iridoid-TIA pathway. Therefore, supplementing the *C. roseus* cell cultures with mevalonic acid might prevent the outflow of MEP pathway intermediates and improve C5 availability for carotenoids and monoterpenoids biosynthesis.

The objective of this study was to evaluate the effect of mevalonic acid feeding on *C. roseus* cell metabolism with specific attention to the distribution of C5-units into representative terpenoid groups: sterols (triterpenoid, C30), carotenoids (tetraterpenoid, C40), and TIAs (monoterpenoid, C10). In this study, we want to see if adding mevalonic acid could change the distribution of C5 precursor in terpenoid groups and if there is indirect evidence of exchange of C5 precursors.

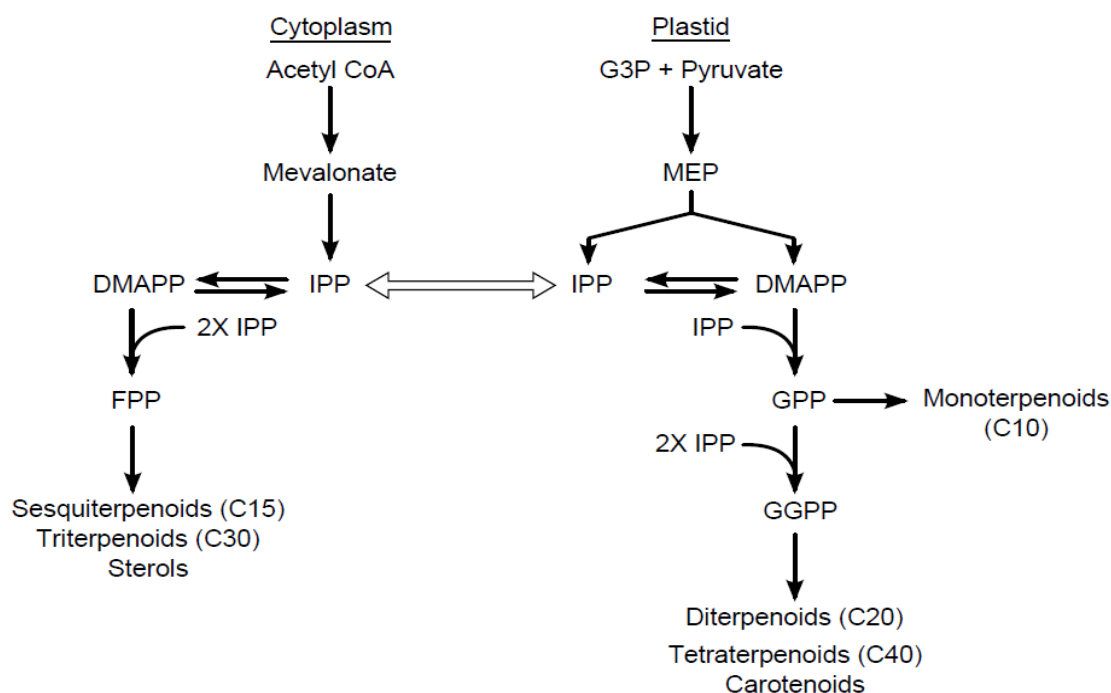


Fig. 1 Mevalonate pathway and 2-C-methyl-D-erythritol 4-phosphate pathway leading to different terpenoid groups. IPP: isopentenyl diphosphate, DMAPP: dimethylallyl diphosphate, GPP: geranyl diphosphate, FPP: farnesyl diphosphate, GGPP: geranylgeranyl diphosphate.

Materials and Methods

Cell culture materials

Cell suspension cultures of *Catharanthus roseus* (cell-line CRPP) were used in this study and the cultivation conditions are described in **Chapter 3**. For the experiment, 20 ml (ca. 4 g fresh weight) of two-week-old cell suspension cultures were inoculated into 50 ml fresh culture medium and maintained under the standard cultivation conditions for 4 days prior to the feeding experiment.

Mevalonic acid preparation

Mevalonic acid was prepared by addition of 130 mg of mevalonolactone (Sigma-Aldrich, St. Louis, MO, USA) to 5.5 ml of 0.2 N KOH. The solution was heated at 50 °C for 15 min. After cooling down to room temperature, the solution was adjusted to pH 7.3 with 0.1 N HCl. Water was added to the solution to achieve the total volume of 10 ml (Popjak 1969). The solution was sterilized through a 0.22 µm MillexTM filter (Millipore, Bedford, MA, USA). Control solution was prepared with the same solvent without mevalonic acid.

Mevalonate feeding and metabolite analysis

Mevalonic acid solution was fed into the 4-day-old cell suspension cultures to achieve final concentrations of 0.2 mM (low mevalonate feeding) and 3.3 mM (high mevalonate feeding). The corresponding control cultures received the same amounts of solution without mevalonic acid. At selected time-points flasks were harvested in triplicate for control and treated samples; regarding the low-dosage treatment flasks were harvested at 0, 2, 6, 24, 48, and 72 hours after feeding, and for the high-dosage treatment at 24 and 72 hours after feeding. The cells were filtered under reduced pressure, subsequently washed three times with deionized water, and lyophilized for 72 hours. Terpenoid indole alkaloids, carotenoids and phytosteroids were analyzed using high performance liquid chromatography-diode array detector (HPLC-DAD) or gas chromatography-flame ionization detector (GC-FID). For the low-dosage experiment, samples were analyzed from duplicate flasks and triplicate flasks for the high-dosage treatment. In addition, samples of the low-dosage experiment (triplicate flasks) were analyzed by ^1H -NMR for evaluation of metabolomic changes. The analytical methods performed in this study are described in **Chapter 3**.

Statistical analysis

A *t*-test was performed on IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL, USA) to determine statistical differences ($P < 0.05$) for the data from triplicate samples.

Results and Discussion

Metabolite analysis

The *Catharanthus roseus* cell suspension cultures (CRPP cell line) were fed with a low and a high level of mevalonic acid solution to achieve a final concentration of 0.2 and 3.3 mM, respectively. The lower concentration was chosen based on the previous work of Contin (1999) who fed the same concentration of loganic acid into *C. roseus* A11 cell line. The higher concentration (3.3 mM mevalonic acid) was previously applied by Moreno et al. (1993) to the *C. roseus* A12A2 cell line, in which at that level the production of C30 (steroid) biosynthetic pathway was found to be saturated in *Nicotiana tabacum* suspension cultures (Threlfall and Whitehead 1988).

Figure 2 and **3** show sterol levels in *C. roseus* cell suspension cultures at low and high mevalonic acid concentrations. For the low mevalonic acid feeding, there were no differences in both treated and control samples after 24 hours, but after 72 hours the levels of campesterol, β -sitosterol, and stigmasterol were 38%, 45%, and 24% increased, respectively,

in the mevalonate fed-cells compared to the controls. Analysis of the sterol accumulation upon high mevalonic acid feeding shows that after 24 hours campesterol and β -sitosterol were 49% and 31% increased, respectively ($P < 0.05$). After 72 hours, the mevalonic acid fed-cells accumulated 126% higher campesterol and 51% higher β -sitosterol levels than the controls ($P < 0.05$). No significant increase in stigmasterol level was observed after 24 hours of high mevalonic acid feeding, whereas 152% increased level was observed 72 hours after the treatment. These results indicate that the exogenously added mevalonic acid affects the sterol levels and distribution in a selective dose and time dependent way in the *C. roseus* cell cultures.

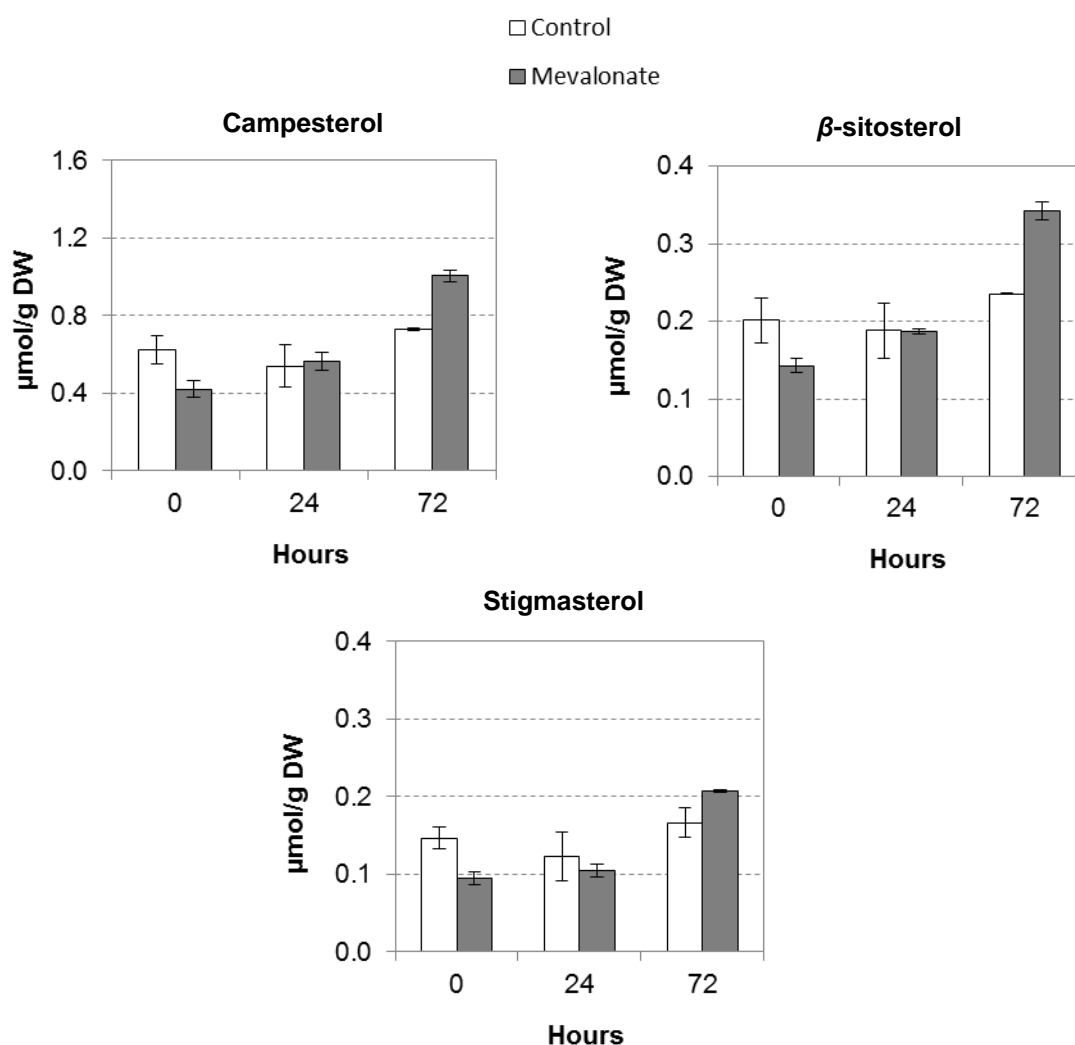


Fig. 2 Sterol production in CRPP cell line fed with a low concentration of mevalonic acid (0.2 mM) or controls measured by GC-Flame Ionization Detector (FID). Results are the mean of two replicates; error bars indicate the two values.

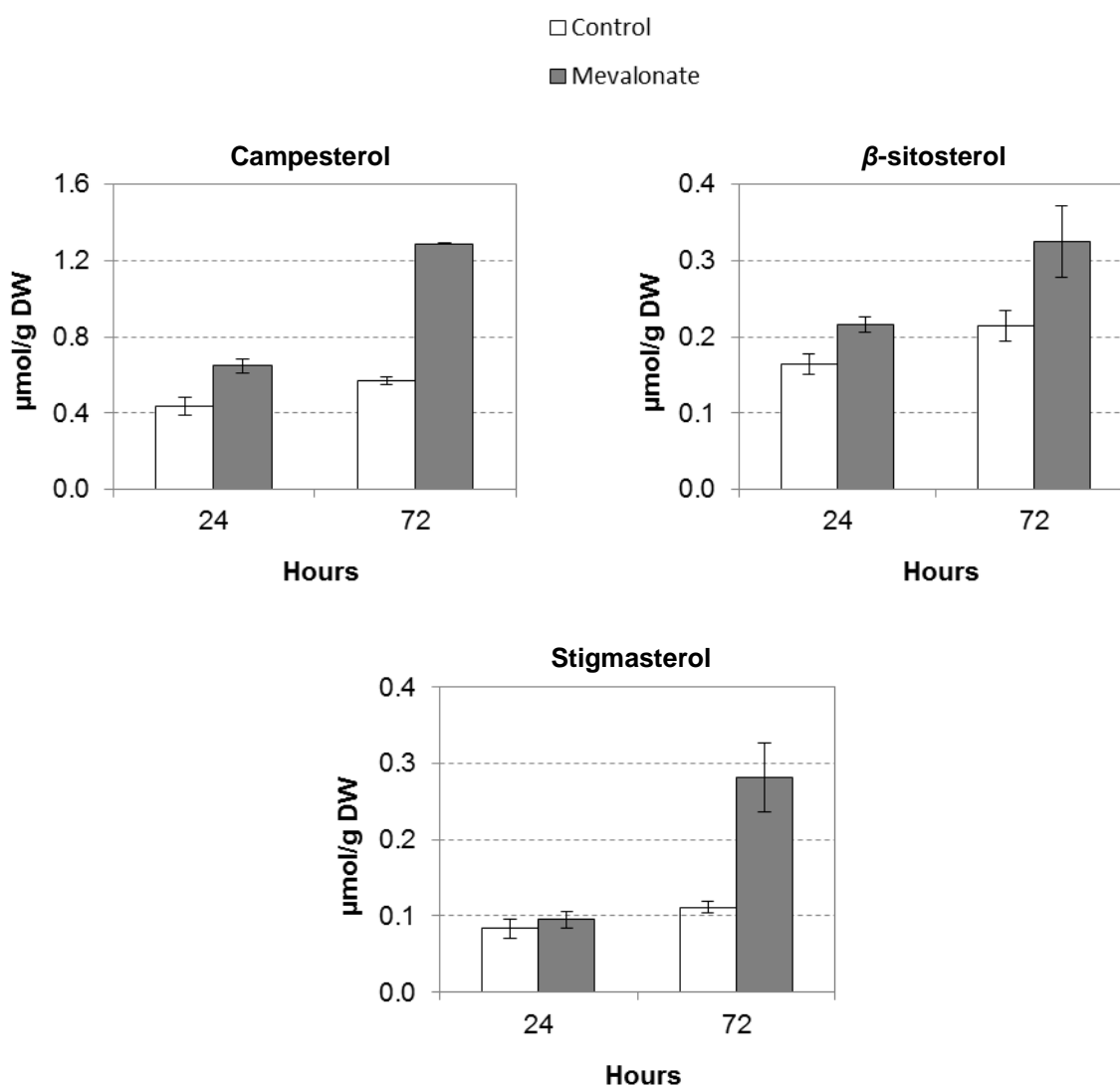


Fig. 3 Sterol production in CRPP cell line fed with a high concentration of mevalonic acid (3.3 mM) or controls measured by GC-Flame Ionization Detector (FID). Results are the mean of three replicates; error bars indicate standard deviation of the mean.

The addition of low or high mevalonic acid feeding did not seem to have any effect on the carotenoid levels compared to their controls (**Fig. 4** and **5**). It is thus presumed that the addition of exogenous mevalonic acid has no or little influence on the flux toward GGPP leading to the carotenoids production under the conditions tested.

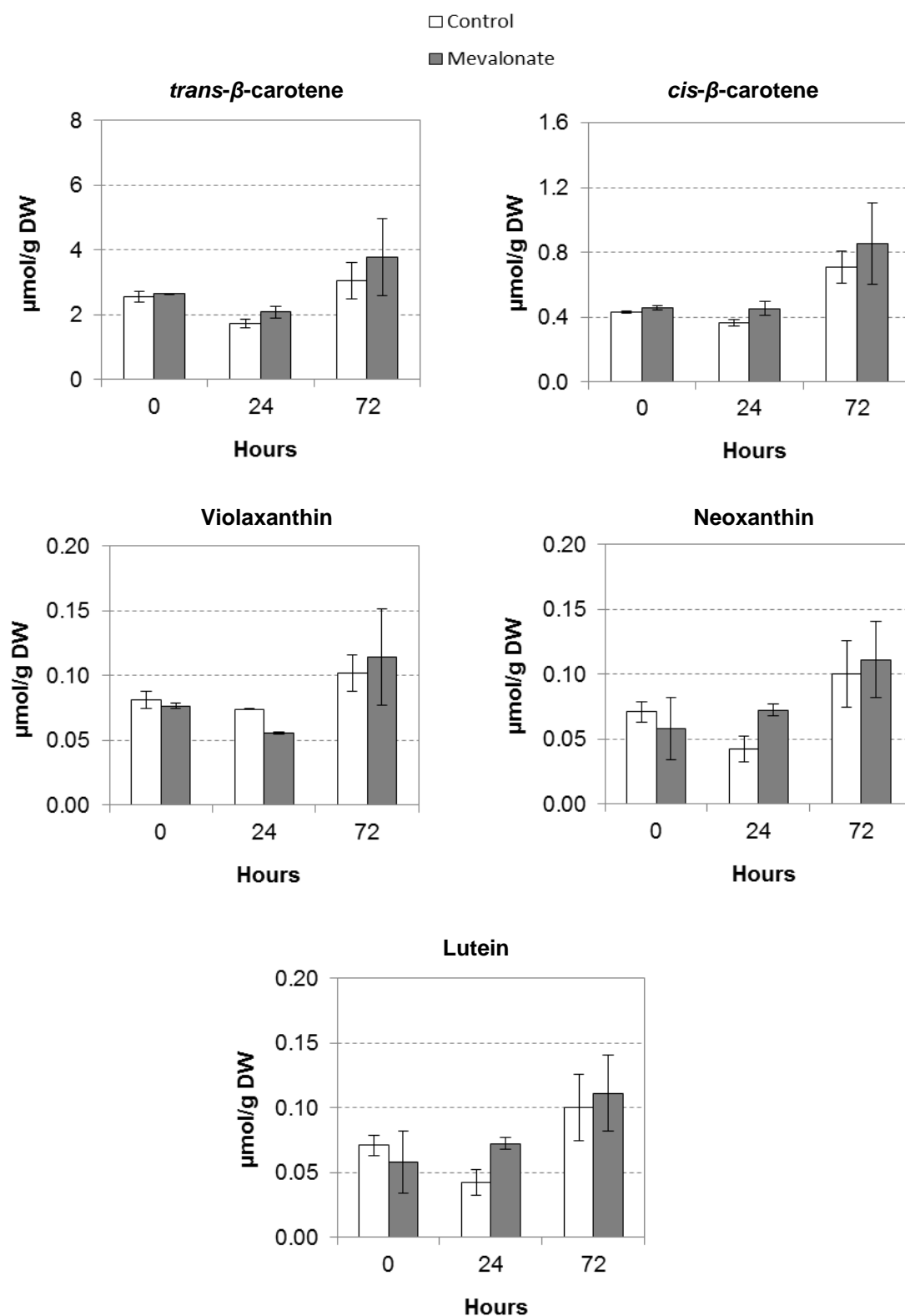


Fig. 4 Carotenoid production in CRPP cell line fed with a low concentration of mevalonic acid (0.2 mM) or controls measured by HPLC-Diode Array Detector (DAD). Results are the mean of two replicates; error bars indicate the two values.

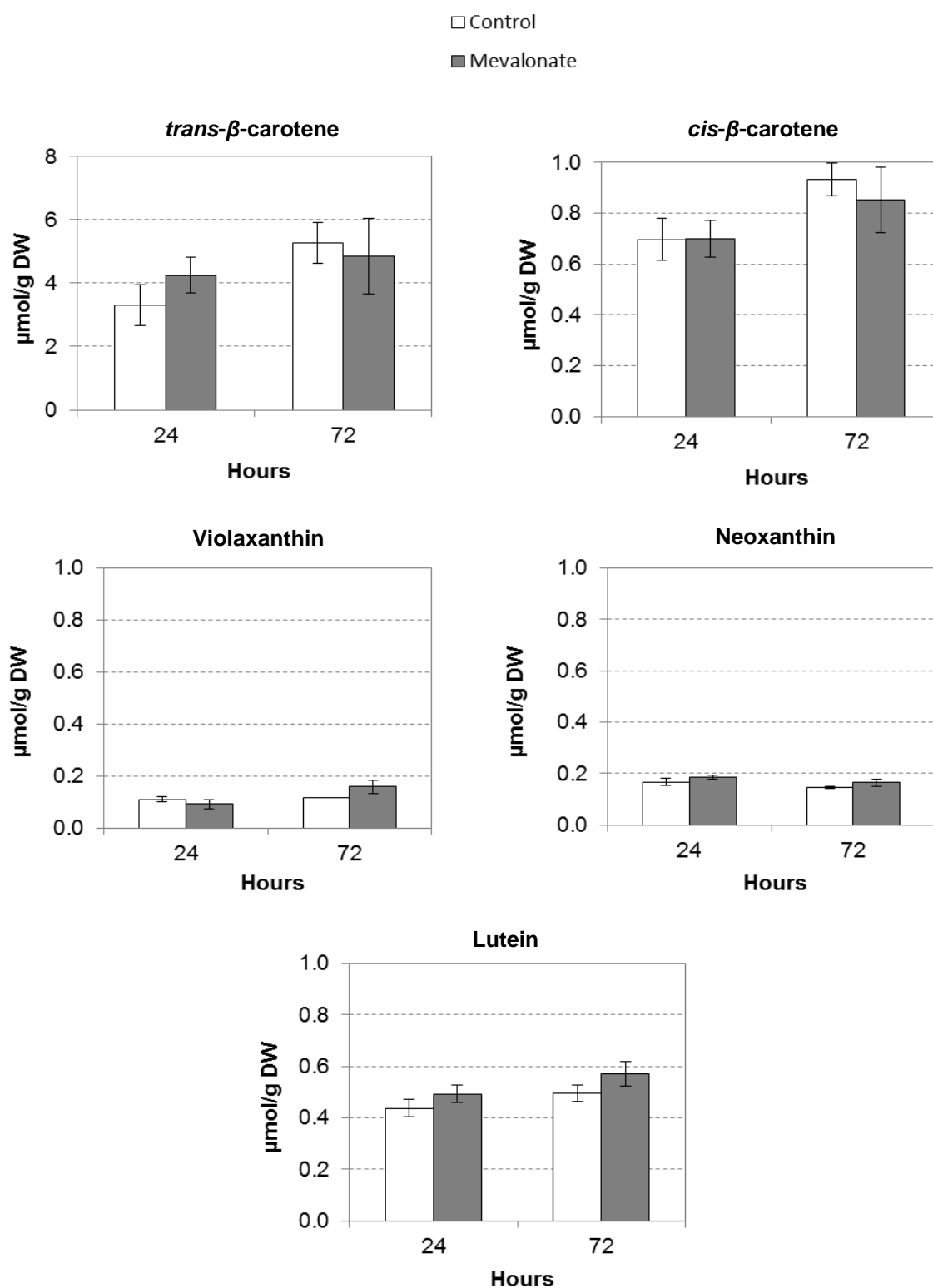


Fig. 5 Carotenoid production in CRPP cell line fed with a high concentration of mevalonic acid (3.3 mM) or controls measured by HPLC-Diode Array Detector (DAD). Results are the mean of three replicates; error bars indicate standard deviation of the mean.

Nevertheless, we noted that both control and treated cultures in the high-mevalonate feeding had a higher carotenoid content than in the low-dosage treatment regarding all individual components. This might be due to a solvent effect or biological variation throughout the cultivation of the cell line. In addition, we observed a pronounced change in the intensity of the green color in the high-mevalonate feeding experiment, as the treated cells turned from green to dark green after 72 hours (**Fig. 6**), which was not observed at low-dosage feeding. Analysis on the chlorophyll levels in the low or high mevalonate feeding did not show any differences between control and the treated cultures (**Fig. 7**). Therefore, the mevalonate-induced color change is neither associated to a difference in carotenoid nor to chlorophyll levels, which leaves the possibility that adjuvants of chlorophylls or carotenoids are affected by mevalonate-derived products and subsequently intensify the color, or that other color components are increased.

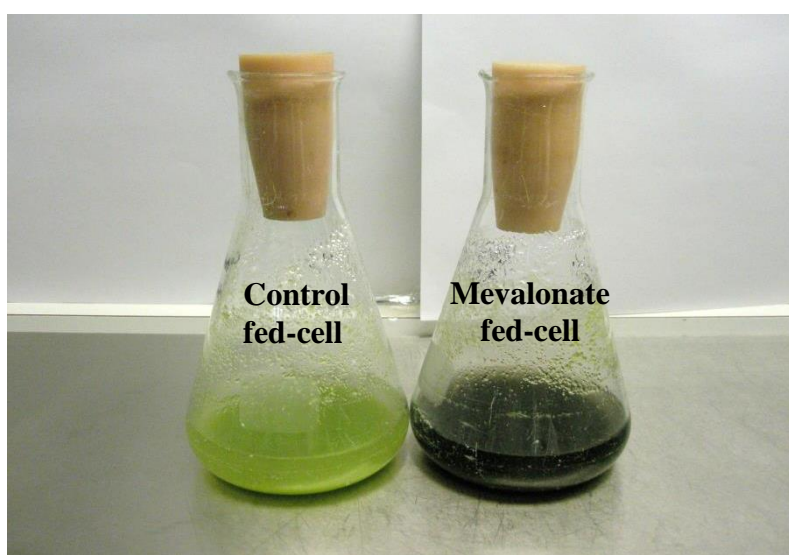


Fig. 6 Comparison of the control and high mevalonic acid fed-cell (3.3 mM) cultures after 72 hours.

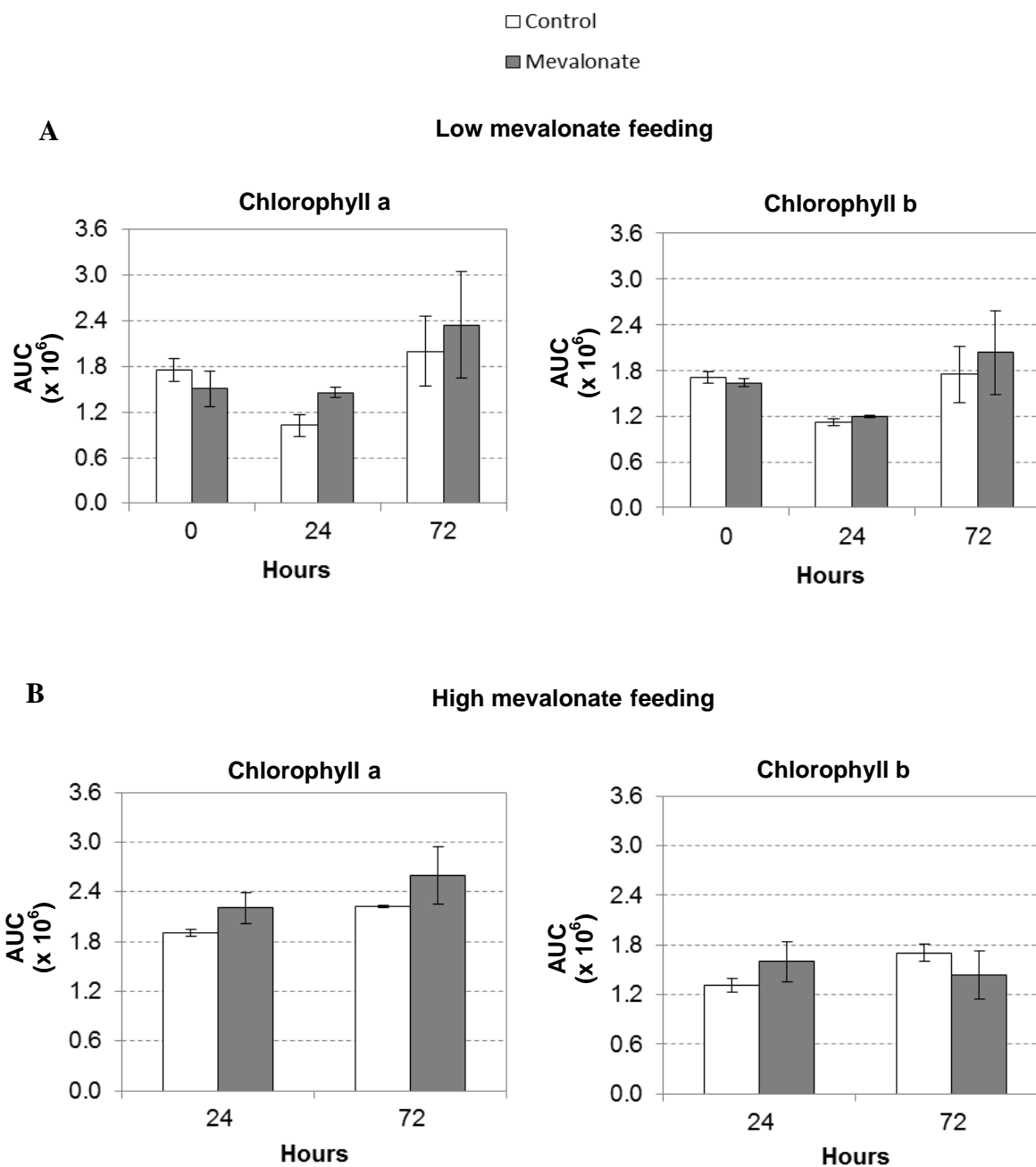


Fig. 7 Chlorophyll levels in CRPP cell line fed with A) low (0.2 mM) or B) high (3.3 mM) mevalonic acid concentration and their respective controls as measured by HPLC-Diode Array Detector (DAD). Results for low mevalonic acid feeding are the mean of two replicates; error bars indicate the two values. Results for high mevalonic acid feeding are the mean of three replicates; error bars indicate standard deviation of the mean. AUC is area under the curve value.

The cell culture used in this study is a TIA-producing *C. roseus* cell line accumulating among others: strictosidine, serpentine, tabersonine, and loganic acid, the iridoid alkaloid precursor from the terpenoid pathway. **Figure 8** shows that the loganic acid production seems to be increased by the low mevalonic acid feeding after 72 hours (twofold higher level; 1.16 $\mu\text{mol/g DW}$), however, the result is not statistically significant due to insufficient sample replicates. The levels of strictosidine, serpentine, and tabersonine were found to be higher in the low mevalonic acid fed-cells compared to the control after 72 hours. However, the levels of strictosidine and serpentine were about the same as at 0 hour, whereas the tabersonine level was lower than at 0 hour. Therefore, the conclusion is that there is no real enhancement in the TIA production after feeding 0.2 mM mevalonic acid to the *C. roseus* cell suspension culture. But it seems that in the controls, TIA levels become lower during time, whereas in the fed cultures it seems to go up. Previous studies by Krueger and Carew (1978), and Moreno et al. (1993) showed no effect on TIA levels in *C. roseus* cell suspension cultures after feeding 0.76 mM and 3.3 mM mevalonic acid, respectively. A similar result was also found by Morgan and Shanks (2000) who fed 52 μM – 104 μM of mevalonic acid to the 17 – 21 days old *C. roseus* hairy root cultures.

A different result was found for loganic acid and TIA levels at the higher concentration of mevalonic acid (3.3 mM) (**Fig. 9**). A twofold increase of loganic acid was observed after 24 hours of mevalonic acid treatment, however TIA levels, i.e. strictosidine, serpentine, and tabersonine were not significantly different compared to the control at that time-point ($P < 0.05$). Subsequently, the levels of loganic acid, strictosidine, and tabersonine dropped below the control level ($P < 0.05$), while tryptamine accumulated (3.5 $\mu\text{mol/g DW}$) in the mevalonate fed-cells after 72 hours (**Fig. 10**). In addition, nologanin and secologanin accumulated in the treated cells. As alkaloid levels are not increased if compared to the controls, the accumulation of tryptamine in the cell is probably due to the lower supply of secologanin to form strictosidine associated to the decreased level of loganic acid after 72 hours treatment. This indicates an adverse/negative effect of the abundant supply of mevalonic acid on loganic acid production. Interestingly, serpentine level was not affected which could be due to the fact that the anhydronium alkaloid serpentine is too polar to be excreted from the vacuoles, and thus will not be catabolized. The results in this study are different from those reported by Moreno et al. (1993) who found insignificant changes of strictosidine and ajmalicine levels at 72 and 120 hours after feeding 3.3 mM of mevalonic acid. The contradictory results could be due to the different cell lines employed in both studies. Saiman et al. (2014) showed that accumulation of terpenoid groups in *C. roseus* cell

suspensions can vary greatly dependent on the specific cell line, which is related to a pronounced difference in the expression of most relevant associated pathway genes in the cell lines.

Courdavault et al. (2005a, 2005b) showed that protein prenylation is involved in the induction of the expression of some of the early stage of monoterpenoid biosynthetic pathway genes (i.e. *DXS*, *DXR*, *G10H* [or *G8O*]), while it has no effect on *SLS*, *TDC*, and *STR* transcript levels. As this prenylation is connected to the mevalonate pathway, it would be interesting to further study the effect of mevalonate feeding on the induction of the activity of these enzymes in the cell culture.

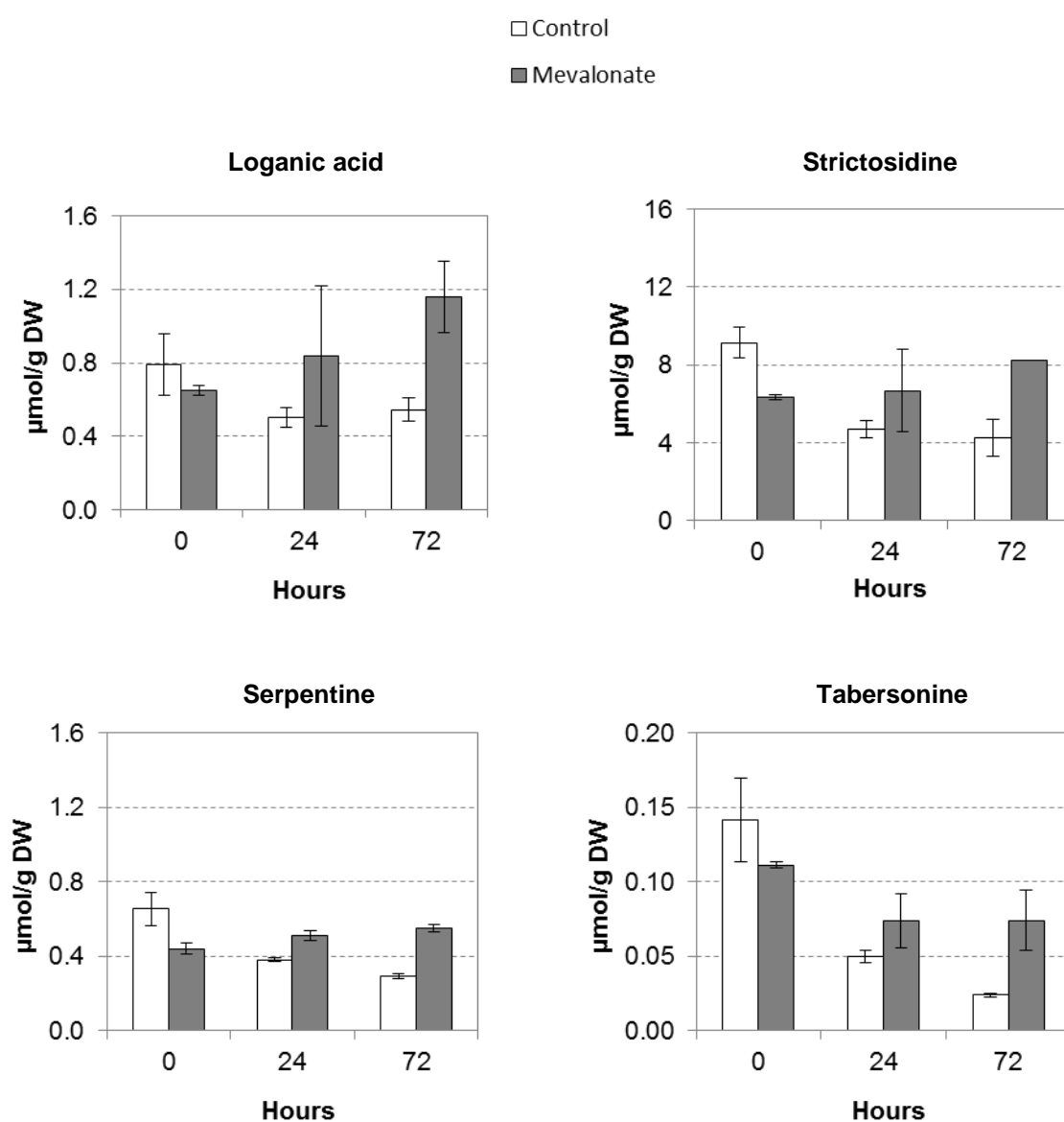


Fig. 8 Loganic acid and terpenoid indole alkaloid production in CRPP cell line fed with a low concentration of mevalonic acid (0.2 mM) or controls measured by HPLC-Diode Array Detector (DAD). Results are the mean of two replicates; error bars indicate the two values.

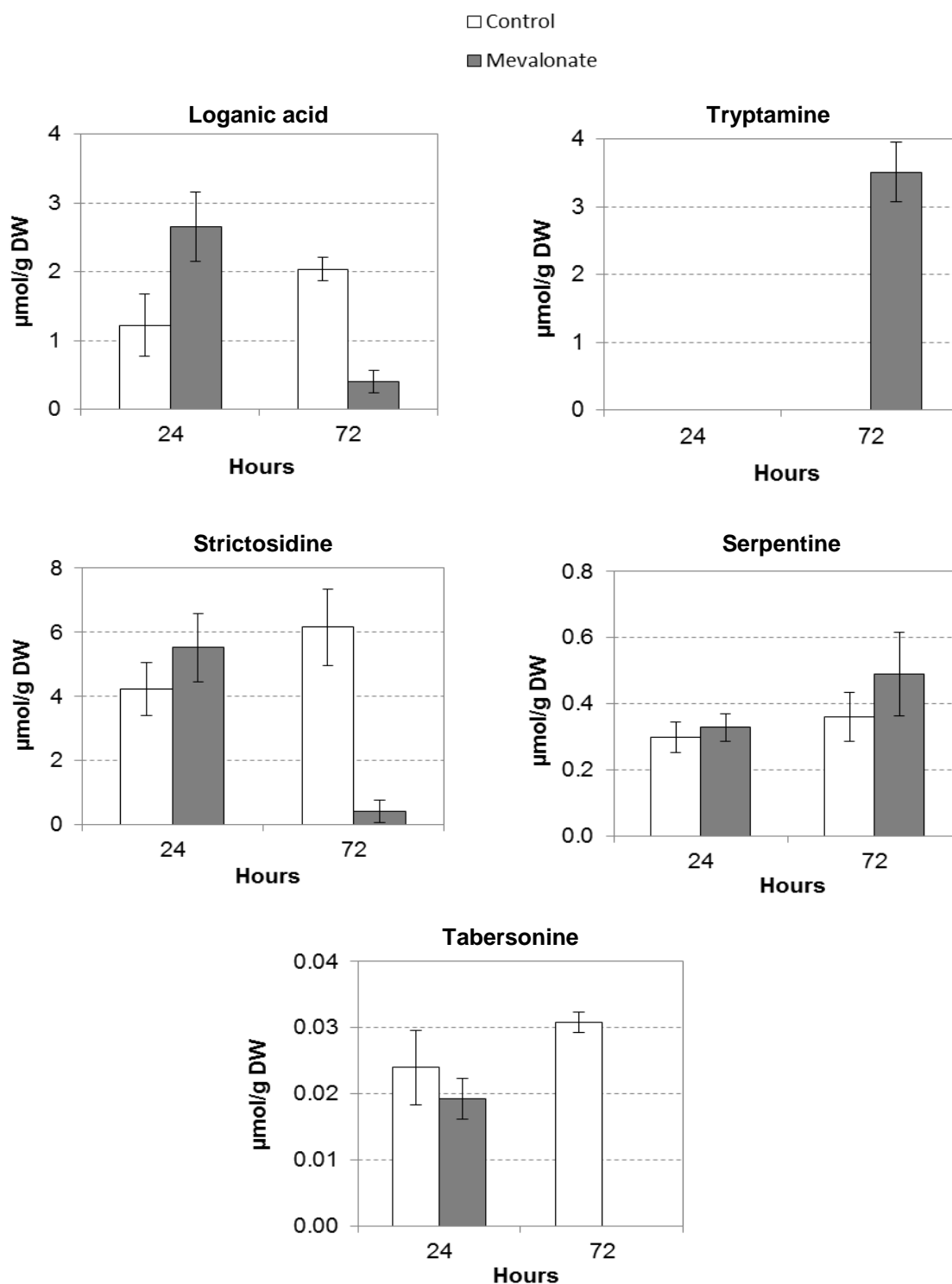


Fig. 9 Loganic acid and terpenoid indole alkaloid production in CRPP cell line fed with a high concentration of mevalonic acid (3.3 mM) or controls measured by HPLC-Diode Array Detector (DAD). Results are the mean of three replicates; error bars indicate standard deviation of the mean.

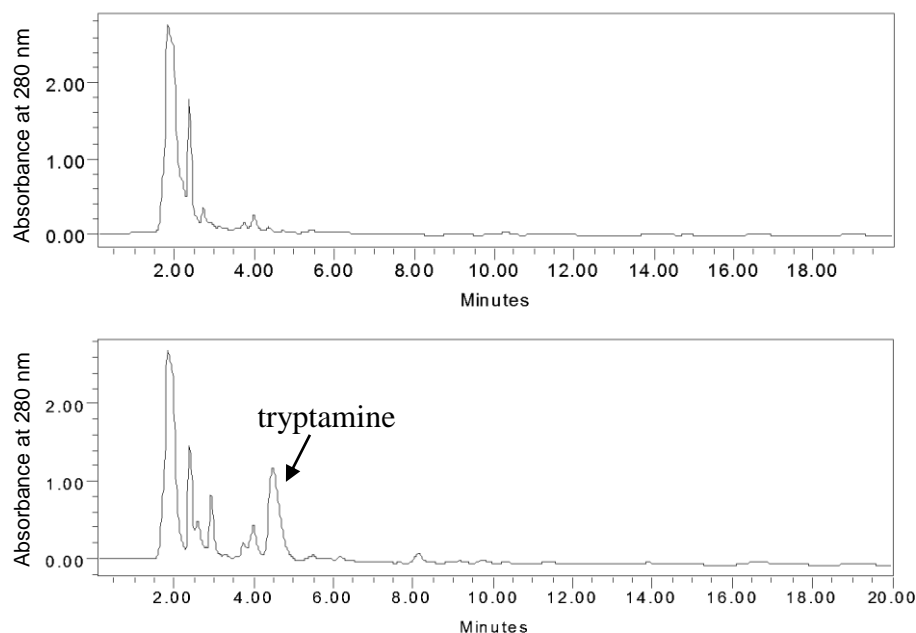


Fig. 10 Chromatograms of high performance liquid chromatography-diode array detector (HPLC-DAD) of control (top) and high mevalonic acid fed-cells (bottom) measured at 280 nm UV wavelength. Tryptamine signal was detected in the high mevalonic acid fed-cells (3.3 mM) after 72 hours.

Total accumulation and C5 distribution

The levels of sterols, carotenoids, and TIAs measured in this study and the distribution of the C5 precursors into the respective groups of metabolite are presented in **Fig. 11**. Apparently, feeding the precursor mevalonic acid to the *C. roseus* cell suspension culture resulted in an increase of sterols. Low mevalonic acid concentration (0.2 mM), resulted in 37% increase of total sterols level after 72 hours compared to the control. In the high mevalonate feeding (3.3 mM), total sterols level was already 40% higher in the treated cells at 24 hours and increased to 111% after 72 hours compared to the control ($P < 0.05$). In the *C. roseus* cell culture, campesterol was found to be the highest accumulated phytosterol, which is in accordance with the report of Suzuki et al. (1995). Feeding mevalonic acid at low concentration maintained campesterol as the major sterol. Campesterol was found as the major sterol (68%) compared to the sum of β -sitosterol and stigmasterol (32%) in the high mevalonic acid feeding at 24 hours after treatment and the ratio was maintained after 72 hours.

As compared to the terpenoid groups measured in this study, the C5 distribution was largest towards the carotenoid production. However, no significant difference of carotenoids level and C5 distribution was observed after mevalonic acid feeding. Consequently, there is

no indirect evidence that the added mevalonic acid reduces an outflow of MEP pathway intermediates (IPP and DMAPP) that might limit carotenoid production. There is no clear enhancement of TIA after low mevalonate feeding as the level was about the same as at 0

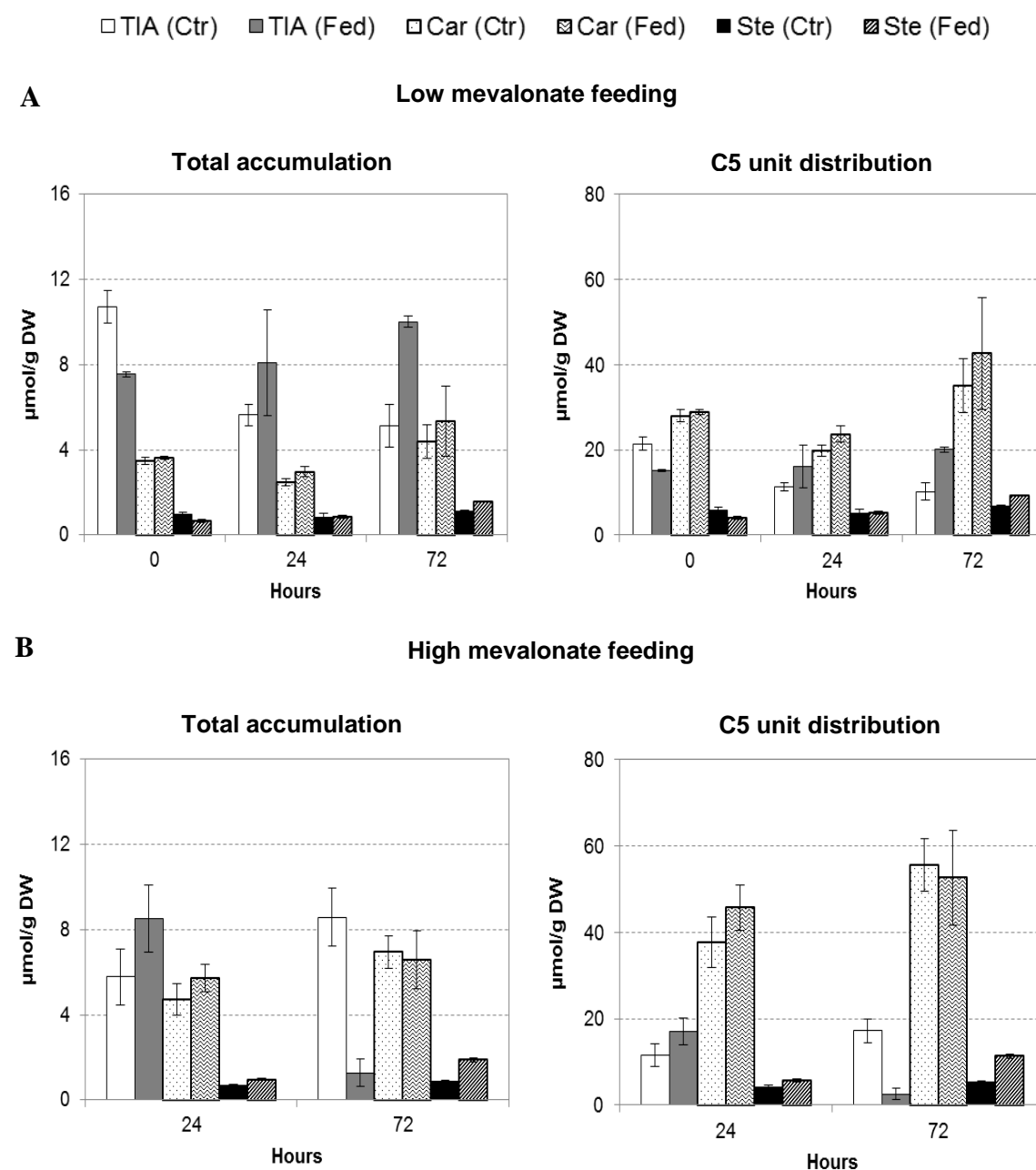


Fig. 11 Total accumulation of terpenoid indole alkaloids, carotenoids, and sterols and the C5 precursor distribution in CRPP cell line fed with A) low (0.2 mM) or B) high (3.3 mM) mevalonic acid concentration and their respective controls. Results for low mevalonic acid feeding are the mean of two replicates; error bars indicate the two values. Results for high mevalonic acid feeding are the mean of three replicates; error bars indicate standard deviation of the mean.

hour. Whereas, an increased the mevalonate concentration (3.3 mM) significantly decreased the TIA level after 72 hours ($P < 0.05$). It is clear that the added mevalonic acid precursor is distributed towards the sterol pathway.

¹H-NMR and multivariate data analysis

Samples of low mevalonate feeding at 0, 2, 6, 24, 48, and 72 hours after treatment were subjected to ¹H-NMR analysis and multivariate data analysis. The unsupervised clustering method known as principal component analysis (PCA) was performed. **Figure 12** shows the PCA score plot of this study using Pareto method as the scaling technique. The samples were separated based on the cell age: 0 – 24 hours samples were separated on the positive side of PC1, while 48 – 72 hours samples were separated on the negative side of PC1. However, no clear separation between the mevalonate fed cells and their controls at different time points is shown in the PCA. This indicates that the differences due to cell age are larger than due to the feeding. Therefore, another technique called partial least squares-discriminant analysis (PLS-DA) was applied to the same bucketed ¹H-NMR spectra. In contrast to PCA which projected the maximum variation within all the samples, PLS-DA is a supervised multivariate data analysis which searches for the differences between defined classes; in this case mevalonate fed cells and controls. As for PCA, the PLS-DA did not exhibit a valid model that could distinguish between the mevalonate feeding and the control samples. In addition, a PLS-DA analysis using only 48 – 72 hours samples did not show separation between the treated and control samples either. This indicates that the profiles of metabolites extracted with methanol-phosphate buffer solvent employed for NMR sample preparation do not significantly differ for the low mevalonate treated-cells and the control samples, and thus that the low mevalonate feeding (0.2 mM) does not have a major effect on the metabolism of the cells. The high mevalonate treated-cells (3.3 mM) will be analyzed to evaluate further metabolic changes upon a high mevalonic acid feeding.

Conclusion

The results obtained in this study show that feeding either a low or a high concentration of mevalonic acid to the TIA-accumulating *C. roseus* cell suspension culture (CRPP line) do not increase the absolute levels of TIA or carotenoids in the MEP pathway. Moreover, the TIA levels at high mevalonate feeding are strongly repressed at a point upstream of loganic acid. The production of sterols derived from the mevalonate pathway is significantly increased after addition of the mevalonic acid in a dose dependent way.

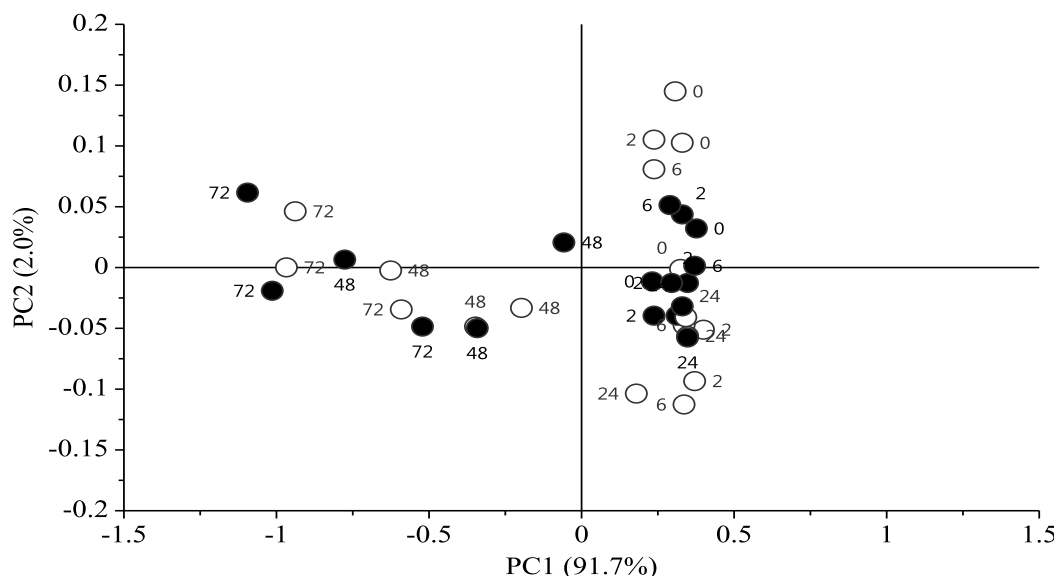


Fig. 12 Score plot of principal component analysis (PCA) of low mevalonic acid fed-cell (●) and control (○) samples of the CRPP cell line measured by ^1H -NMR. The numbers in the score plot are harvesting time (hour) after treatments.

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