Chapter 5

# Effect of geraniol feeding and jasmonic acid elicitation on terpenoid indole alkaloids accumulation in *Catharanthus roseus* cell suspension cultures

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**Abstract**

*Catharanthus roseus* is an important medicinal plant producing terpenoid indole alkaloids (TIA). Previous studies showed that geraniol may be limiting as an upstream precursor in the monoterpenoid pathway. Therefore, in the present study, geraniol was fed to *C. roseus* cell suspension cultures (cell-line CRPP) at increasing concentrations (0.32 – 1.62 mM). After feeding, the cell cultures were found to accumulate loganic acid (up to 10-fold) and a large new peak was detected by HPLC-DAD, in a concentration- and time-dependent manner. However, the production of strictosidine and down-stream TIA seemed unaffected upon geraniol feeding, whereas at the higher geraniol feeding concentrations (1.30 – 1.62 mM) catharanthine and tabersonine levels decreased compared to the control cultures. As determined for this cell line, JA elicitation increased strictosidine, serpentine, tabersonine, catharanthine, and loganic acid levels. The latter compound is significantly increased only at 24 hours after treating with lower JA concentration (34 and 102 μM), but did not accumulate to the level as detected by geraniol feeding. A combination treatment of geraniol feeding (1.62 mM) at day 4 and JA elicitation (102 μM) at day 5 showed a significant increased level of strictosidine (68%) compared to the single JA treatment at 72 hours after elicitation. However, the combination treatment did not further improve serpentine production while catharanthine and tabersonine levels were lower like in case of high geraniol feeding. These results show that feeding geraniol to *C. roseus* cell suspension cultures leads to accumulation of loganic acid, and in combination with JA elicitation the flux towards strictosidine is increased, but not to further down-stream TIA like serpentine, catharanthine, and tabersonine. Furthermore, an improved HPLC-DAD method showed 5 new peaks in the geraniol-fed cells, the NMR spectra of the extract showed several signals similar to the proton signals as geraniol and thus point to the presence of geraniol analogues. Nevertheless, due to the complexity of the mixture, isolation of the compounds is required for further identification.

**Introduction**

*Catharanthus roseus* is a rich source of terpenoid indole alkaloids (TIA). It is one of the most extensively studied medicinal plants and consequently served as an important model system for plant biotechnology and secondary metabolites production (van der Heijden et al. 2004). The TIA are products of condensation from two biosynthetic routes; tryptamine, derived from the amino acid tryptophan (one of the products of the shikimate pathway), and secologanin derived from geraniol (product of the monoterpenoid pathway) (van der Heijden

et al. 2004). The latter is known to be synthesized from the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (Contin et al. 1998).

Precursor feeding studies were performed in *C. roseus* cell cultures to determine the metabolic flux limitations in the TIA biosynthesis. The addition of loganic acid, loganin, or secologanin increased TIA levels in the *C. roseus* cell cultures and thus it was suggested that the TIA production in *C. roseus* cell is limited by availability of precursors in the monoterpenoid pathway (Moreno et al. 1993; Whitmer et al. 1998, 2002). Studies were also conducted to observe the effect of feeding early precursors in the monoterpenoid pathway. A study by Krueger and Carew (1978) showed that feeding geraniol, the upstream precursor of the iridoid moiety produced from geranyl diphosphate (GPP), did not affect the TIA production in *C. roseus* cell cultures. In contrast, geraniol treatment reduced growth and TIA production in compact callus clusters cultures of *C. roseus* (Zhao et al. 2001). However, Morgan and Shanks (2000) reported that feeding geraniol increased the tabersonine level in *C. roseus* hairy root cultures and Lee-Parsons and Royce (2006) also found the increase of ajmalicine production in geraniol-fed *C. roseus* cell suspension cultures. The latter results suggested that geraniol may be the limiting factor as upstream precursor of the monoterpenoid pathway.

In this study, the transient effect of geraniol feeding on the production of TIA and iridoid precursors in the *C. roseus* cell-line CRPP was analyzed. Furthermore, the effect of jasmonic acid in the geraniol-fed CRPP cell cultures was assessed to determine how jasmonate elicitation alters the precursor availability for TIA biosynthesis. Experiments were also conducted at increasing concentration of geraniol and jasmonic acid to determine the appropriate concentration treatment.

## Materials and methods

### *Cell materials*

*Catharanthus roseus* cell suspension cultures (cell-line CRPP-glucose) were grown in B5 medium (Gamborg et al. 1968) supplemented with 20 g/l glucose and 1.86 mg/l 1-naphthaleneacetic acid (NAA). The cell suspension cultures were placed on a gyratory shaker (110 rpm) under continuous light (10.8 – 27 µE/$m^2$/sec) at 25 ± 1 $^oC$. To obtain sufficient biomass for the experiment, the CRPP cell line was subcultured every two weeks by subculturing 1:1 dilution of 100 ml culture into 100 ml fresh medium and equally divided to two flasks. For inoculation, two to three flasks of the two-week-old CRPP cell cultures were

randomly mixed together in one flask and subsequently 20 ml of the cell suspensions (approx. 4 g FW cells) were pipetted into 50 ml of fresh medium.

*Preparation of geraniol and jasmonic acid solutions*

Geraniol (Sigma-Aldrich, St. Louis, MO, USA) was diluted in 50% v/v ethanol to make a 100 mg/ml stock solution. The stock solution of jasmonic acid (10 mg/ml) was prepared by dissolving jasmonic acid (Sigma-Aldrich) in 2 ml ethanol and diluted with sterile water to acquire the total volume of 10 ml. The stock solutions were filter-sterilized using 0.22 µm Millex$^{TM}$ filters (Millipore, Bedford, MA, USA).

*Different concentrations of geraniol feeding and jasmonic acid elicitation*

Geraniol stock solution was added to the cultures on the fourth day after inoculation to deliver end concentrations of 0.32, 0.65, 0.97, 1.30, and 1.62 mM geraniol. Jasmonic acid stock solution was added on the fifth day after inoculation to deliver final concentrations of 34, 102, 170, and 238 µM jasmonic acid. The same amount of solvent used in geraniol feeding (50% v/v ethanol) and jasmonate elicitation (20% v/v ethanol) were applied to the cell cultures as control. All treatments were done in triplicate flasks and cultures were harvested after 24 and 72 hours.

*Geraniol feeding and combination of geraniol feeding and jasmonic acid elicitation*

For geraniol feeding, the cell cultures on the fourth day after inoculation were fed with geraniol stock solution to achieve the final concentration of 1.62 mM. The cells were harvested at 0, 24, 48, and 96 hours after treatment. For feeding + elicitation, the cultures fed with geraniol on the fourth day were elicited with jasmonic acid on the fifth day after inoculation to the final concentration of 102 µM. The cells were harvested at 0, 24, and 72 hours after elicitation. The same amounts of solvent used in geraniol feeding and jasmonate elicitation were applied to the cell cultures as solvent control. Control blank cultures without any treatment were also collected at all harvesting time-points. Each treatment was performed in triplicate.

*Harvesting*

Samples were harvested using a Bűchner vacuum-filtration unit and rinsed with deionized water. Subsequently, the cells were immediately frozen in liquid nitrogen and

lyophilized for 72 hours to deliver biomass accumulation in dry weight (DW) per flask. Samples were kept at room temperature in the dark until further analysis.

*TIA and precursors analysis*

Terpenoid indole alkaloids (strictosidine, ajmalicine, serpentine, catharanthine, tabersonine, vindoline, vinblastine, vincristine) and precursors (loganic acid, loganin, secologanin, tryptophan, tryptamine) were extracted and analyzed by high performance liquid chromatography-diode array detector (HPLC-DAD) methods as described by Saiman et al. (2014). The HPLC-DAD methods were run on an Agilent Technologies 1200 series chromatograph equipped with a diode array detector and a Zorbax Eclipse XDB-C18 column (250 mm x 4.6 mm, particle size 5 µl) (Agilent Techologies Inc., Santa Clara, CA, USA).

Separation of new precursor peaks (A – E) was carried out using a 150 x 4.6 mm Luna 5 micron C18 (2) 100A column (Phenomenex Inc., Torrance, CA, USA) and performed on the same HPLC-DAD series (Agilent Techologies Inc.). The mobile phase consisted of acetonitrile (A) and 0.1% trifluoroacetic acid in water (B). The eluent profile (volume of solvent A/volume of solvent B) was: 0 – 5 min, isocratic elution with 10:90 (v/v); 5 – 30 min, linear gradient from 10:90 to 95:5 (v/v); 30 – 35 min, isocratic elution with 10:90 (v/v). The flow rate was 1.0 ml/min and the injection volume was 10 µl. The diode array detector was set at 240 nm with a UV spectrum scan from 190 – 390 nm.

*Geraniol analysis*

The dried cells (100 mg) were extracted two times with 5 ml ethyl acetate, vortexed, sonicated for 20 min, and centrifuged at 3500 rpm (4 $^{o}$C) for 10 min. The supernatants were combined and concentrated to dryness under reduced pressure. The residue was dissolved in 400 µl methanol. For culture medium, 5 ml liquid medium was extracted with 5 ml ethyl acetate, vortexed, and centrifuged at 3500 rpm (4 $^{o}$C) for 10 min. This procedure was repeated three times. The combined supernatants were concentrated to dryness and the residue was dissolved in 400 µl methanol. The samples were filtrated over miracloth prior to geraniol analysis.

Geraniol analysis was carried out using an Agilent 6890 series (Agilent Technologies Inc.) gas chromatograph (GC) equipped with 7863 series injector, a DB-5 capillary column (30 m x 0.25 mm i.d., film thickness of 0.25 um) (J&W Scientific Inc., Folsom, CA, USA) and coupled with a flame ionization detector (FID). Analysis conditions were: injector temperature: 230 $^{o}$C, detector temperature: 250 $^{o}$C, initial oven temperature: 100 $^{o}$C. A

temperature gradient was applied running from 100 $^{o}$C to 140 $^{o}$C at 10 $^{o}$C/min and then from 140 $^{o}$C to 240 $^{o}$C at 35 $^{o}$C/min, and maintaining 240 $^{o}$C for 20 min. Nitrogen was used as carrier gas (15.5 psi) at 1.2 ml/min flow rate and the injection split ratio was 1 to 50. The sample injection volume was 5 µl. Identification of the products was performed by comparison with standard compounds.

*NMR analysis*

NMR analysis was conducted according to the protocol of Kim et al. 2010. Fifty milligrams of freeze-dried cells were extracted with 1.2 ml of methanol-$d_4$ and 0.3 ml of potassium dihydrogen phosphate buffer in deuterium oxide (pH 6). The mixture was vortexed for 10 sec, sonicated for 10 min, and centrifuged for 15 min (14,000 rpm). An aliquot of 0.8 ml of the supernatant was transferred into an NMR tube for measurement using a Bruker AV 600 MHz spectrometer (Bruker, Karlsruhe, Germany) with cryoprobe. The $^{1}$H-NMR spectra were recorded at 25 $^{o}$C, consisted of 128 scans requiring 10 min and 26 sec acquisition time with following parameters: 0.16 Hz/point, pulse width of 30 (11.3 µs), and relaxation delay of 1.5 sec. Methanol-$d_4$ was used as the internal lock. A presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay. Free induction decay was Fourier transformed with a line-broadening (LB) factor of 0.3 Hz. The resulting spectra were manually phased, baseline corrected, and calibrated to trimethylsilyl propionic acid sodium salt (TMSP-$d_4$) at 0.0 ppm by using XWIN NMR version 3.5 (Bruker).

Two-dimensional *J*-resolved NMR spectra were acquired using 8 scans per 64 increments for F1 and 1,638.4 k for F2 using spectral widths of 6,009.6 Hz in F2 (chemical shift axis) and 50 Hz in F1 (spin–spin coupling constant axis). A 1.5 sec relaxation delay was employed. Datasets were zero filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex FT. The *J*-resolved spectra were tilted by 45$^{o}$, symmetrized about F1, and calibrated to TMSP- $d_4$ using XWIN NMR version 3.5 (Bruker). The $^{1}$H–$^{1}$H correlated spectroscopy (COSY) spectra were acquired with a 1.0 sec relaxation delay and 6,009.6 Hz spectral widths in both dimensions. The window function for the COSY spectra was sine-bell (SSB = 0). The HMBC spectra were obtained with 1.0 sec relaxation delay, 30183 Hz spectral width in F2 and 27164 Hz in F1. Qsine (SSB = 2.0) was used for the window function of the HMBC.

*Statistical Analysis*

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

**Results and discussion**

*Evaluating different concentrations of geraniol feeding and jasmonic acid elicitation*

The effects of various concentrations of geraniol and jasmonic acid in *Catharanthus roseus* cell culture (cell-line CRPP) were studied by analyzing the cell dry weight and terpenoid indole alkaloids (TIA) production including iridoid precursor accumulation. Two separate studies were conducted by feeding various end concentrations of geraniol (0.32 – 1.62 mM) and jasmonic acid (34 – 238 µM) to the cell cultures. Feeding at the final concentration of 0.32 – 1.30 mM geraniol to the *C. roseus* cell cultures showed that the cell biomass yields were quite stable and comparable to the controls (**Fig. 1**). However, cultures treated at a final geraniol concentration of 1.62 mM had a slightly lower biomass than their corresponding controls. Morgan and Shanks (2000) indicated that there were toxic effects on the growth of *C. roseus* hairy roots when geraniol was fed to the cultures at 160 mg/l (1.04 mM) or higher concentrations. Furthermore, Carriere et al. (1989) reported that the cell viability of *C. roseus* and three other plant cell cultures remained higher than 80% at 24 hours after feeding 100 mg/l geraniol (0.65 mM), but decreased rapidly beyond this concentration.

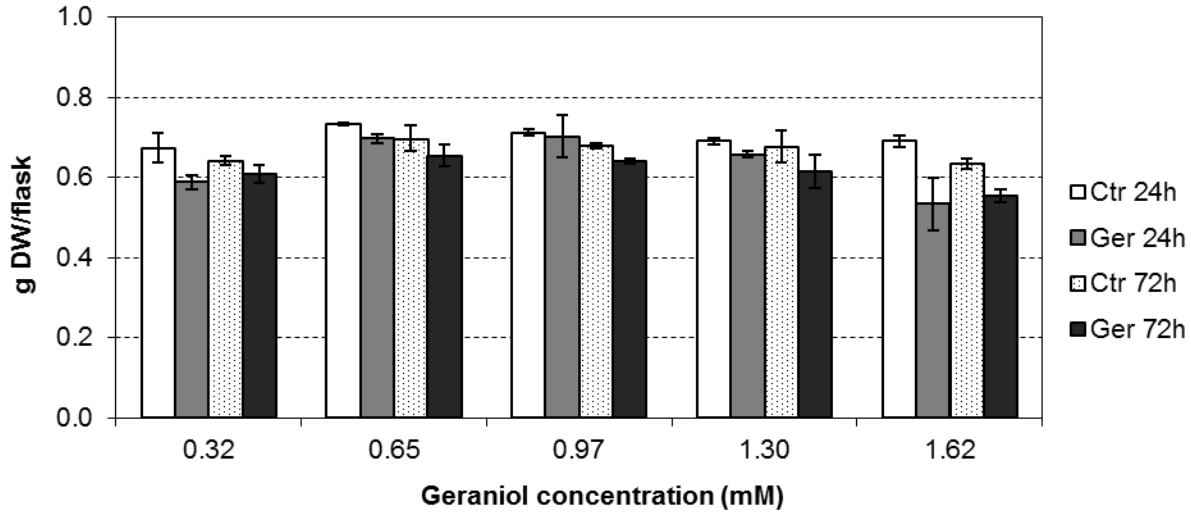


**Fig. 1** Cell biomass of *Catharanthus roseus* cell culture (cell-line CRPP) treated with different concentrations of geraniol. Error bars represent standard deviations obtained from three replicates.

In the present study, the CRPP biomass accumulation (g dry weight/flask) was reduced to 23% after feeding geraniol at 1.62 mM, yet the appearance of the cultures seemed similar in density and color (green) to the solvent controls. Therefore, we concluded that it is safe to apply between 50 and 250 mg/l geraniol (0.32 – 1.62 mM) to the CRPP cell cultures without inducing strong adverse effects on biomass accumulation.

The production of TIA and TIA precursors of iridoids (i.e. loganic acid, loganin, and secologanin), tryptophan, and tryptamine was analyzed at 24 and 72 hours after feeding different concentrations of geraniol (0.32 – 1.62 mM) in the *C. roseus* cell cultures (cell-line CRPP). No tryptophan, tryptamine, loganin, and secologanin were detected in the cell cultures, only loganic acid was found (**Fig. 2**). The level of loganic acid increased in a geraniol concentration dependent manner by 2- to 10-fold at 24 hours after geraniol feeding; the maximal increase obtained was 10-fold with feeding 1.30 mM geraniol compared to control ($P < 0.05$). At 72 hours after feeding, the levels of loganic acid were consistently lower than at the 24-hour time-point, but still 2- to 3-fold higher than the corresponding controls; this loganic acid accumulation pattern suggests uptake and conversion of geraniol to loganic acid in the first 24 hours, and subsequent metabolization to other products. In addition, there is accumulation of a new peak of unknown compound observed at 240 nm at the retention time about similar to secologanin that only appeared upon feeding with geraniol. However, detailed analysis of the UV spectrum showed that the peak had a maximum UV absorption at 224 nm instead of the maximum at 238 nm for secologanin (**Fig. 3**). The level of this compound increased in a geraniol concentration dependent manner. The highest level of this unknown peak was obtained at feeding of 1.62 mM geraniol with a 2.6-fold increase at 72 hours compared to 24 hours after treatment (AUC from 4558 to 11989).

Although the level of precursors was increased after geraniol feeding, the results showed that the level of the first alkaloid strictosidine was not affected except a decrease at 24 hours after treatment with 1.62 mM geraniol, which was half of the control. Furthermore, serpentine levels were not significantly different after geraniol feeding as compared to the controls at 24 and 72 hours after treatment ($P < 0.05$). Lee-Parsons and Royce (2006) also found no significant difference in serpentine levels between control and geraniol-fed cells (0.5 mM) after 48 hours, even though ajmalicine content was slightly increased. The levels of catharanthine and tabersonine were decreased in the geraniol-fed cells at 1.30 and 1.62 mM compared to the controls ($P < 0.05$). Apparently, an increased iridoid concentration is not immediately reflected in an increased alkaloid concentration, which could be due to the lack

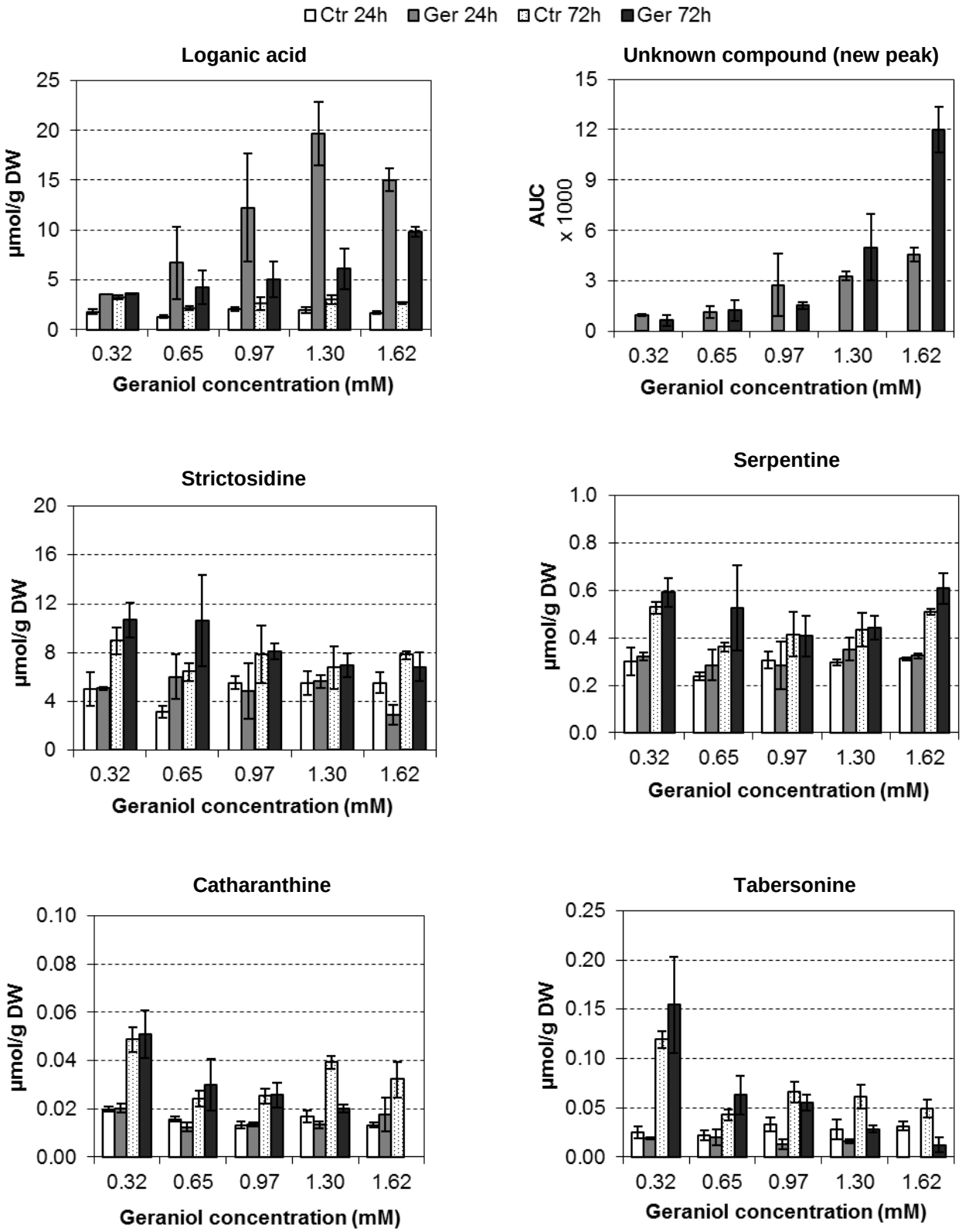


**Fig. 2** Accumulation of some compounds in *Catharanthus roseus* cell culture (cell-line CRPP) treated with different concentrations of geraniol. Error bars represent standard deviations obtained from three replicates.

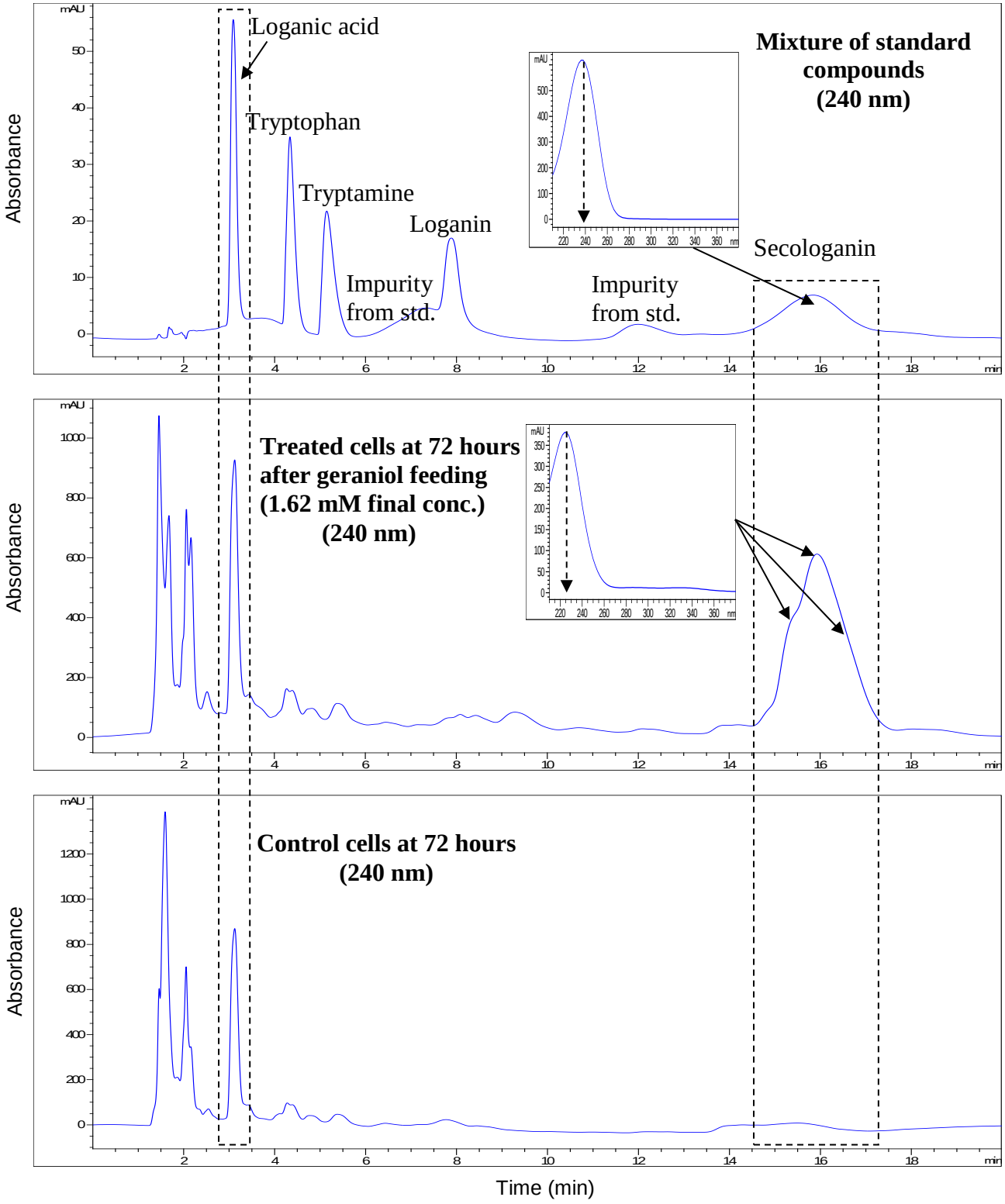


**Fig. 3** Chromatograms of HPLC-Diode Array Detector (DAD) of mix standard compounds, geraniol-fed (1.62 mM) *Catharanthus roseus* cells, and control cells at 240 nm. A huge unknown peak is present in geraniol-fed cells at the retention time and UV spectrum close to secologanin.

of tryptamine. The decrease in alkaloid levels at high geraniol level might be due to a stress reaction of the cells resulting in catabolism of alkaloids.

Elicitation of the CRPP cell cultures with different JA concentrations (34 – 238 µM) showed no effect on the cell biomass yield over the tested time-period (**Fig. 4**). A study by Rijhwani and Shanks (1998) showed that final concentrations of 24 – 238 µM JA did not have a significant effect on the biomasses of *C. roseus* hairy root cultures. Therefore, it is presumed that the JA concentration range applied in this study is not toxic to the CRPP cell cultures. **Figure 5** shows the levels of loganic acid and TIA in the jasmonic acid treated-cell cultures. Jasmonic acid elicitation showed a positive effect on loganic acid accumulation but only at the lower concentration of 34 and 102 µM, at 24 hours after treatment ($P < 0.05$). In addition, it is noted that loganic acid did not accumulate to the level as produced after geraniol feeding and the new peak of the unknown compound found in the geraniol feeding experiments was not present in elicitation study (see **Fig. 2** and **3**). Regarding strictosidine, the levels were about twofold increased by JA elicitation both at 24 hours and 72 hours after treatment, and reached a maximum of 18 µmol/g DW upon elicitation. Considering serpentine, the levels were not significantly increased after 24 hours elicitation as compared to their respective controls ($P < 0.05$) except at the 102 µM elicitation treatment. At 72 hours upon elicitation, the average values of serpentine increased 25% – 62%. Although the highest serpentine level was 0.76 µmol/g DW at 72-hour time-point after feeding 102 µM JA, the variation between biological replicates was quite high and therefore the levels is not

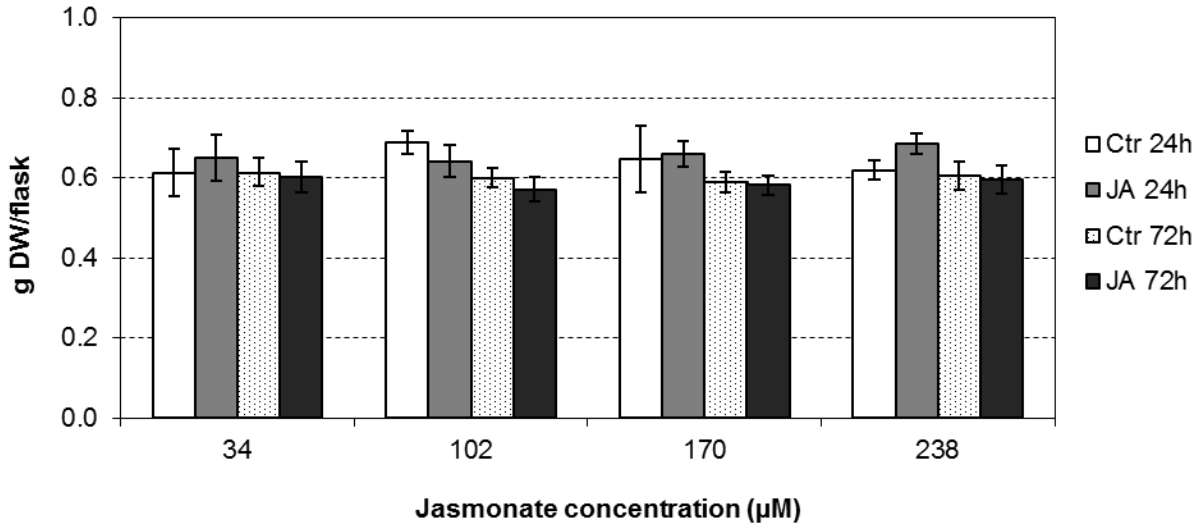


**Fig. 4** Cell biomass of *Catharanthus roseus* cell culture (cell-line CRPP) treated with different concentrations of jasmonic acid. Error bars represent standard deviations obtained from three replicates.

statistically significant different to the control ($P < 0.05$). Tabersonine level had highest increment at 24 hours after elicitation of 102 μM (3.6-fold) and 170 μM (3.8-fold) JA. Catharanthine levels had increased about twofold at 24 hours and 72 hours after 102 μM JA elicitation ($P < 0.05$). These results indicate that the flux of metabolites to TIA is clearly enhanced by JA elicitation. Several studies have shown that exogenous application of jasmonates enhances TIA production (Rijhwani and Shanks 1998; El-Sayed and Verpoorte 2002, 2004; Lee-Parsons et al. 2004; Lee-Parsons and Royce 2006; Vázquez-Flota et al. 2009) and the induction is associated with the elevated expression of a set of genes involved in TIA biosynthesis such as geraniol synthase (Simkin et al. 2013), geraniol 10-hydroxylase or also known as geraniol 8-oxidase (Collu et al. 2001), anthranilate synthase, D-1-deoxyxylulose 5-phosphate synthase, tryptophan decarboxylase, strictosidine synthase, and strictosidine $\beta$-D-glucosidase (Memelink et al. 2001). Our present study also shows that the TIA levels did not significantly increase in a JA concentration dependent way ($P < 0.05$), and that the differences in responses between biological replicates can be quite high. The central TIA intermediate strictosidine is by far the most abundant compound of the TIA pathway. Serpentine is present at about 20 times lower level, whereas tabersonine and catharanthine levels were two orders of magnitude lower. Small changes in strictosidine levels may thus cause larger effects on these minor alkaloids. But in fact the changes observed may in relative terms be similar to those observed for strictosidine, in terms of absolute amounts they represent a very low percentage of the precursor strictosidine.

*Combination treatment of geraniol feeding and JA elicitation*

The combination treatment of geraniol feeding and JA elicitation could affect both precursor availability and TIA formation. In a previous study using the Leiden *C. roseus* A11 cell line, Lee-Parsons and Royce (2006) reported that feeding geraniol (0.5 mM) doubled the ajmalicine level if compared to the non-fed cells. However when feeding geraniol (0.1 – 1.0 mM) a day after elicitation, the ajmalicine production was not significantly affected compared to jasmonate elicited cells. As gene activation and transcription to enzymes occurs within minutes to a few hours upon jasmonate elicitation (Menke et al. 1999, van der Fits and Memelink 2001), the addition of the precursor may result in no significant changes in product accumulation when applied a day after elicitation. Therefore, in the present study we tested the reverse approach by adding jasmonic acid a day (day 5) after geraniol feeding (day 4).

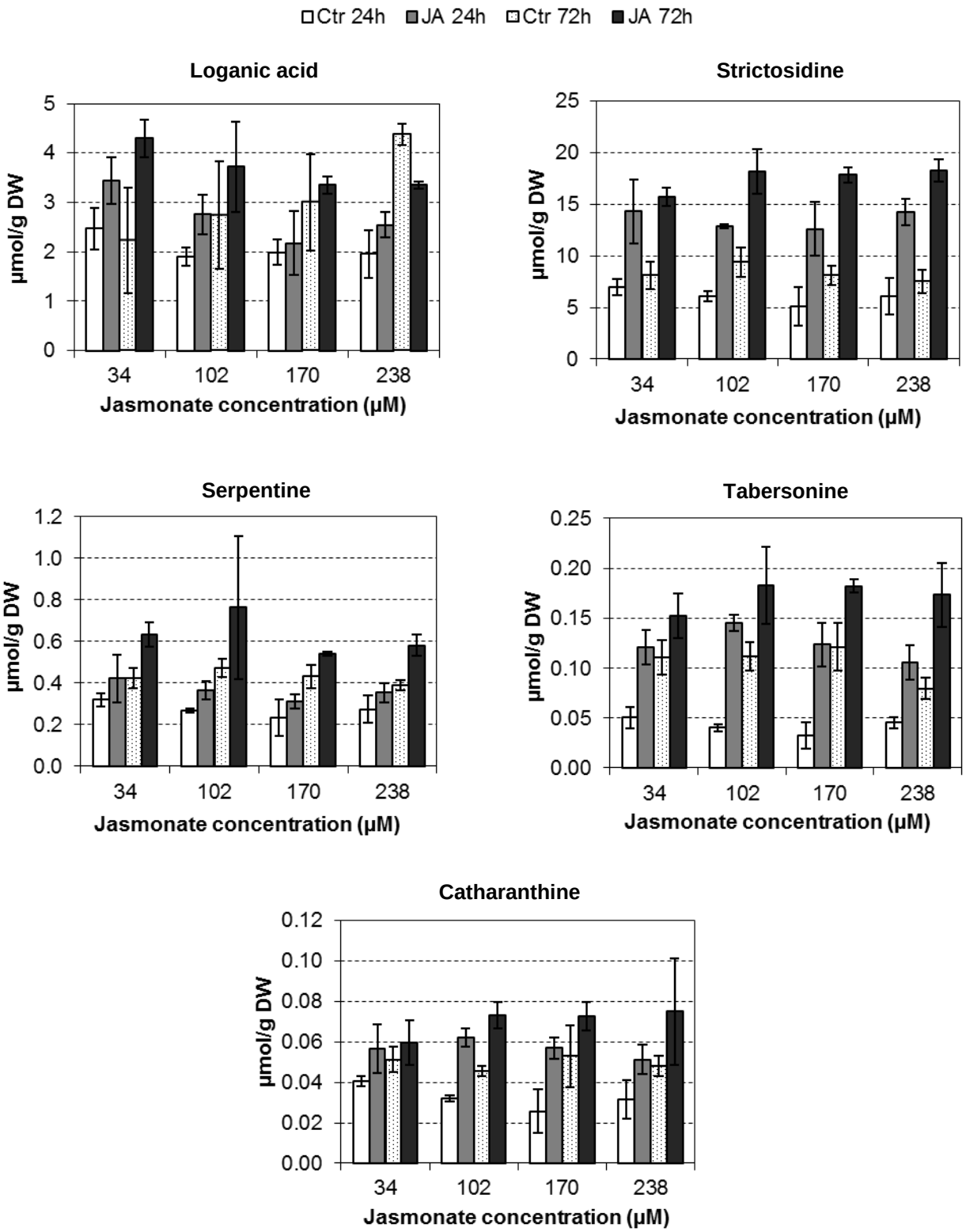


**Fig. 5** Accumulation of some compounds in *Catharanthus roseus* cell culture (cell-line CRPP) treated with different concentrations of jasmonic acid. Error bars represent standard deviations obtained from three replicates.

Based on the earlier experiments, the combination treatment of 1.62 mM geraniol and 102 μM JA concentrations was chosen because that geraniol concentration resulted in high accumulation of loganic acid and the new compound, whereas the concentration of 102 μM JA was used because it seems to have a better induction of TIA accumulation.

**Figure 6** shows the effect of geraniol and jasmonic acid combination treatments on the accumulation of the *C. roseus* cell (cell-line CRPP) biomass. The biomass accumulation was lower after feeding 1.62 mM geraniol (final concentration). At day 8 after inoculation (96 hours after geraniol feeding), the cell DW was 27% and 20% lower in geraniol feeding and geraniol + JA treatment, respectively as compared to their controls. This result shows that the additional JA elicitation does not further reduce growth of the cell cultures beyond the effect of the fed geraniol, as discussed in the previous paragraph. Despite the reduced biomass accumulation, the appearance of the geraniol-fed cell cultures with or without added JA seemed comparable to the control cultures, as mentioned before for the geraniol treatment. Analysis of geraniol in the *C. roseus* culture medium showed that it was no longer present in the liquid medium after 24 hours (**Supplement 1**), while also no geraniol was detected in the cell cultures at any of the time-point. The rapid disappearance of geraniol from the liquid medium of cell cultures in 24 hours is also found by Carriere et al. (1989), who indicated that the disappearance of geraniol is mainly due to metabolization into other products rather than being lost by evaporation. The combination treatment confirms the capacity of the CRPP cells to take up and metabolize a high concentration of geraniol without strong adverse effects on biomass accumulation, and this makes it a useful model cell line to study the geraniol and iridoid pathways, including the effects of signaling molecules like JA.

**Figure 7** shows the effects of geraniol and the combination treatment with jasmonic acid on loganic acid and TIA at different time-points. Loganic acid was clearly increased (8- to 10-fold compared to the control) at 24 hours (day 5) after geraniol feeding ($P < 0.05$). At day 6, loganic acid remained at high level in the geraniol-fed cells, while it decreased to control levels in the geraniol-fed cells treated with jasmonic acid at 24 hours after elicitation. Strictosidine production was not affected in geraniol feeding, but upon subsequent JA elicitation, strictosidine was increased at 24 hours after elicitation compared to the solvent controls and reached the highest level (30.6 μmol/g DW) after 72 hours (day 8). Though a precise mass balance cannot be made, the decrease in loganic acid is followed by an increase of strictosidine in geraniol + JA treatment. Interestingly, the increasing level in geraniol + JA treatment was 68% higher ($P < 0.05$) than after the single JA elicitation treatment (18.2

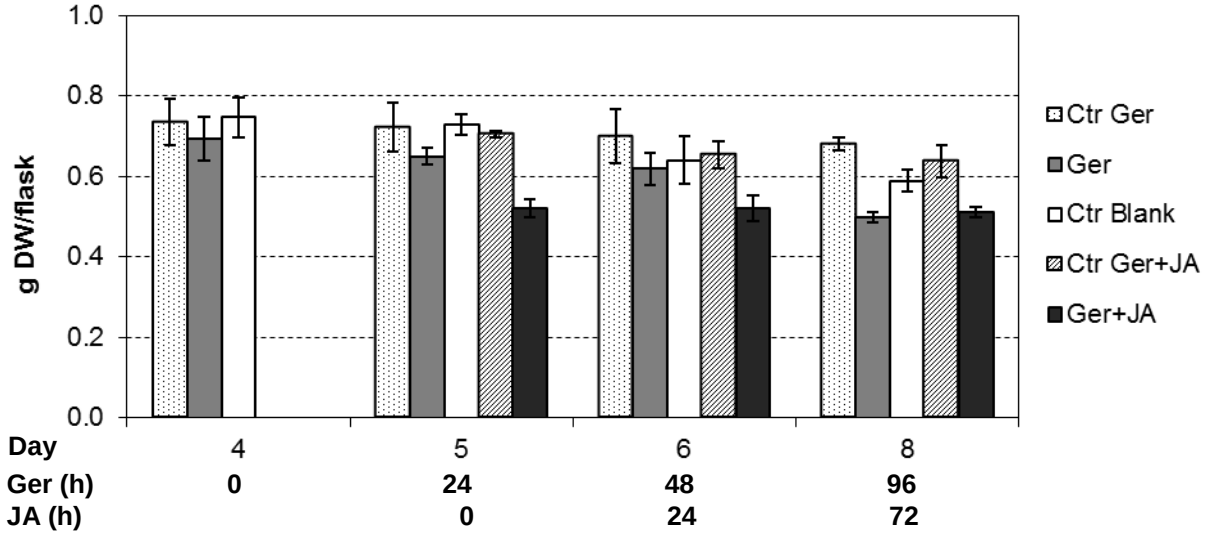


**Fig. 6** Cell biomass of *Catharanthus roseus* cell culture (cell-line CRPP) treated with final concentration of 1.62 mM geraniol (Ger) at day 4 and in combination with 102 µM jasmonic acid (JA) added at day 5. Error bars represent standard deviations obtained from three replicates.

µmol/g DW) at the same concentration (102 µM) after 72 hours (see **Fig. 5**). This result shows that a combination of geraniol + JA elicitation specifically increases the flux towards strictosidine. It should be noted that even though strictosidine level did not increase in the only geraniol feeding treatment, the loganic acid accumulation was decreased to the level of the control at day 8 (after 96 hours), suggesting catabolism of loganic acid to other products.

Serpentine levels were not affected significantly after feeding geraniol but they were increased 72 hours after geraniol-fed cells were elicited with jasmonic acid ($P < 0.05$). The serpentine level (0.6 µmol/g DW) at day 8 (72 hours after elicitation) was comparable to the level of serpentine in the single jasmonic acid treatment (see **Fig. 5**).

In the initial study using different concentrations of geraniol, feeding at 1.30 mM and 1.62 mM final concentrations significantly reduced the level of catharanthine and tabersonine ($P < 0.05$). In this study, neither of these compounds was detected in the cells at 96 hours (day 8) after geraniol feeding. Furthermore, subsequent JA elicitation of the geraniol-fed cells did not result in detectable tabersonine and catharanthine accumulation, even though only JA elicitation resulted in higher levels of both compounds (**Fig. 5**). In a previous study, Morgan and Shanks (2000) found an increase of tabersonine levels after feeding geraniol to the *C. roseus* hairy roots cultures. However, the results may not be comparable because Morgan and Shanks (2000) used a much lower concentration of geraniol (0.05 – 0.10 mM) and the root

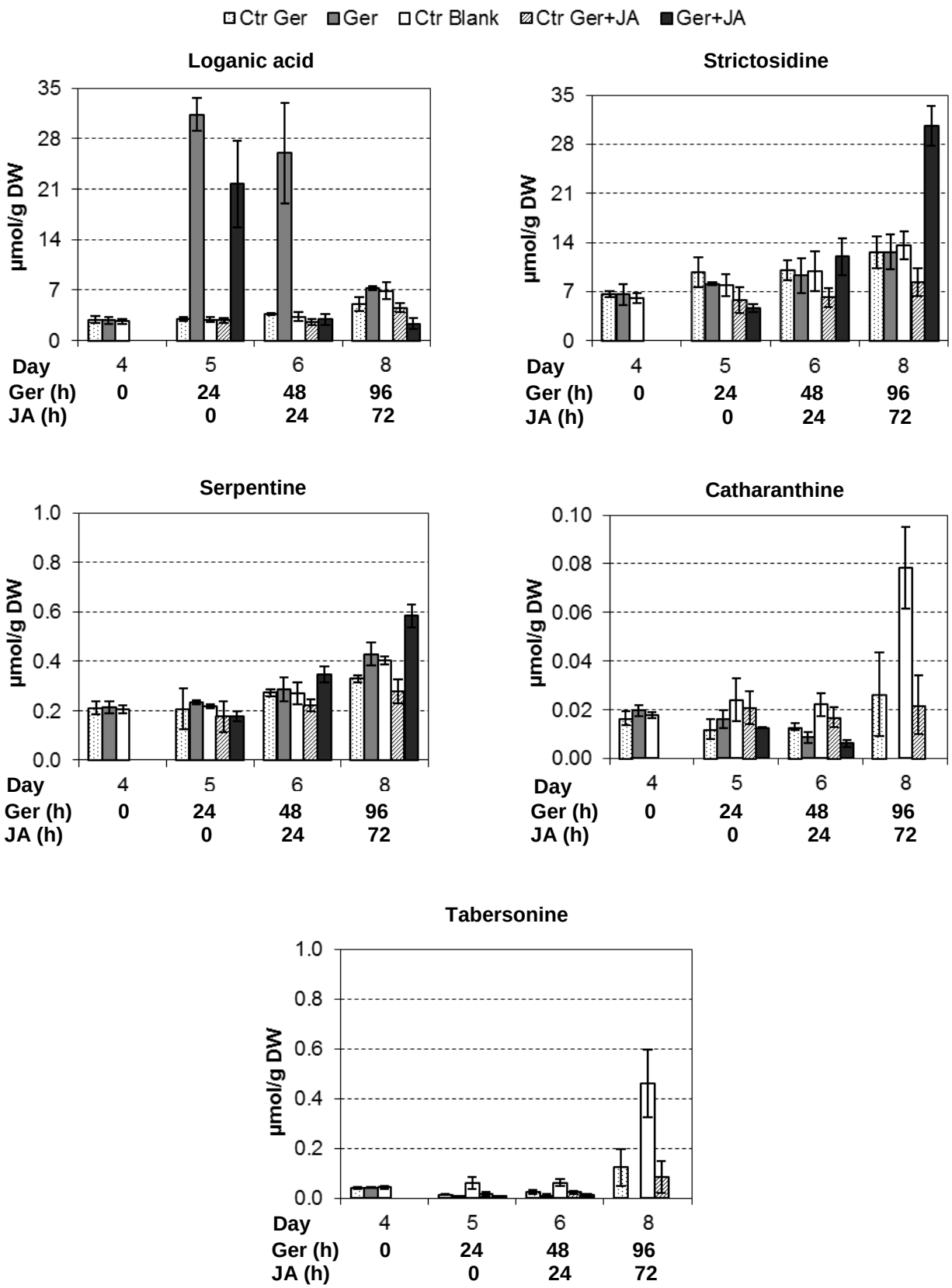


**Fig. 7** Accumulation of some compounds in *Catharanthus roseus* cell culture (cell-line CRPP) treated with final concentration of 1.62 mM geraniol (Ger) at day 4 and in combination with 102 µM jasmonic acid (JA) added at day 5. Error bars represent standard deviations obtained from three replicates.

cultures may have responded differently in their uptake and metabolism than our cell suspension cultures. It may be interesting to test the combination of JA elicitation and lower concentrations of geraniol feeding on the TIA and precursors productions in our CRPP cell-line, in which continuous or repetitive feeding could be considered to deliver equal amounts of carbon units over a longer feeding-period as alternative to the high single spiking approach currently employed.

Apart from a negative influence on tabersonine and catharanthine accumulation by high levels of geraniol, it should be noted that tabersonine and catharanthine levels in the control solvent samples (50% v/v ethanol for geraniol feeding, 50% + 20% v/v ethanol for geraniol + JA treatment) were also lower compared to the control blank samples. This indicates that there is probably an additional effect of ethanol to catharanthine and tabersonine production.

As the changes in loganic acid levels after geraniol feeding was not compensated by an increase of known iridoid intermediates (i.e. loganin and secologanin), strictosidine and down-stream TIA, it was interesting to look for new compounds that appeared upon geraniol feeding. Indeed, there is the large unknown peak detected in the HPLC chromatogram of the geraniol-fed samples at the retention time of secologanin, but it had a slightly different UV absorption spectrum if compared to secologanin (see **Fig. 3**). In this case, another HPLC-DAD method was employed to further analyze the broad peak, which resulted in an improved separation and detection of several new peaks subsequently labeled as A, B, C, D and E (**Fig. 8**). **Figure 9** shows the peaks had different UV absorption spectra if compared to loganic acid, secologanin, and strictosidine. The transient levels of those peaks after feeding geraniol and in combination with JA elicitation are shown in **Fig. 10**. Upon geraniol feeding, peaks A and B increased over time followed by a decrease of loganic acid. However, peaks C and D levels are quite stable over time. The level of peak A and B at JA elicitation was about 2 times lower and higher, respectively, than that of the only geraniol feeding. At 24 hours after JA elicitation, peak A was again about similar to only geraniol feeding, whereas the level of peak B was about double than that of the geraniol feeding but did not increase any further in the next period up to 96 hours. Loganic acid level was remarkably decreased at 24 hours after elicitation and remained at the same level at 96 hours, but this level was about 3 times lower than that of the only geraniol feeding. Peak C was about doubled at JA elicitation compared to the only geraniol feeding, the level maintained at 24 hours but subsequently reduced about half at 72 hours (day 8) compared to the initial level. Peak D was about the same level after

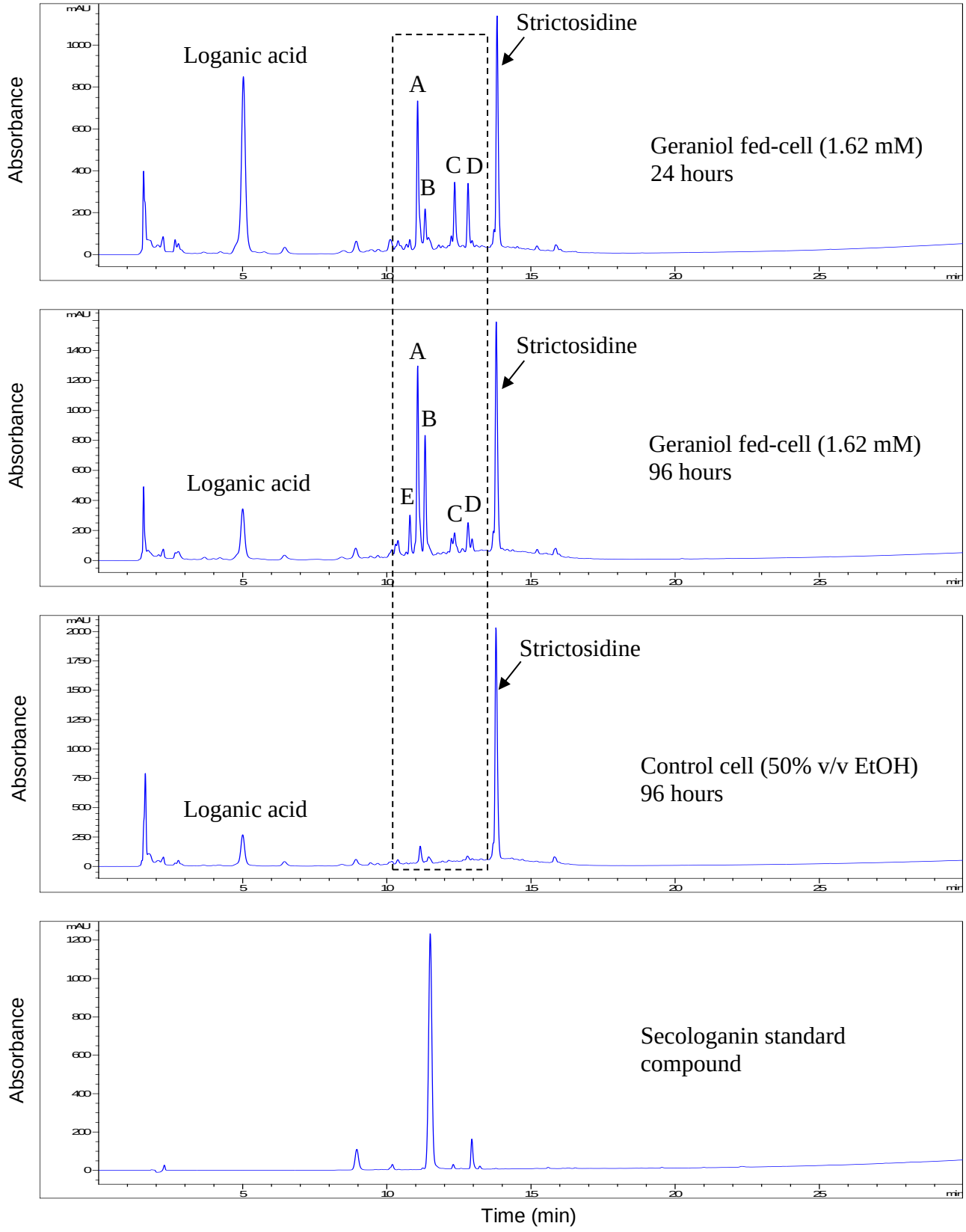


**Fig. 8** Chromatograms of HPLC-Diode Array Detector (DAD) of *Catharanthus roseus* cell culture (cell-line CRPP) at 24 and 96 hours after geraniol feeding (1.62 mM), control cell after 96 hours, and standard compound of secologanin. The new peaks labelled as A, B, C, D, and E is detected in the geraniol-fed cells.

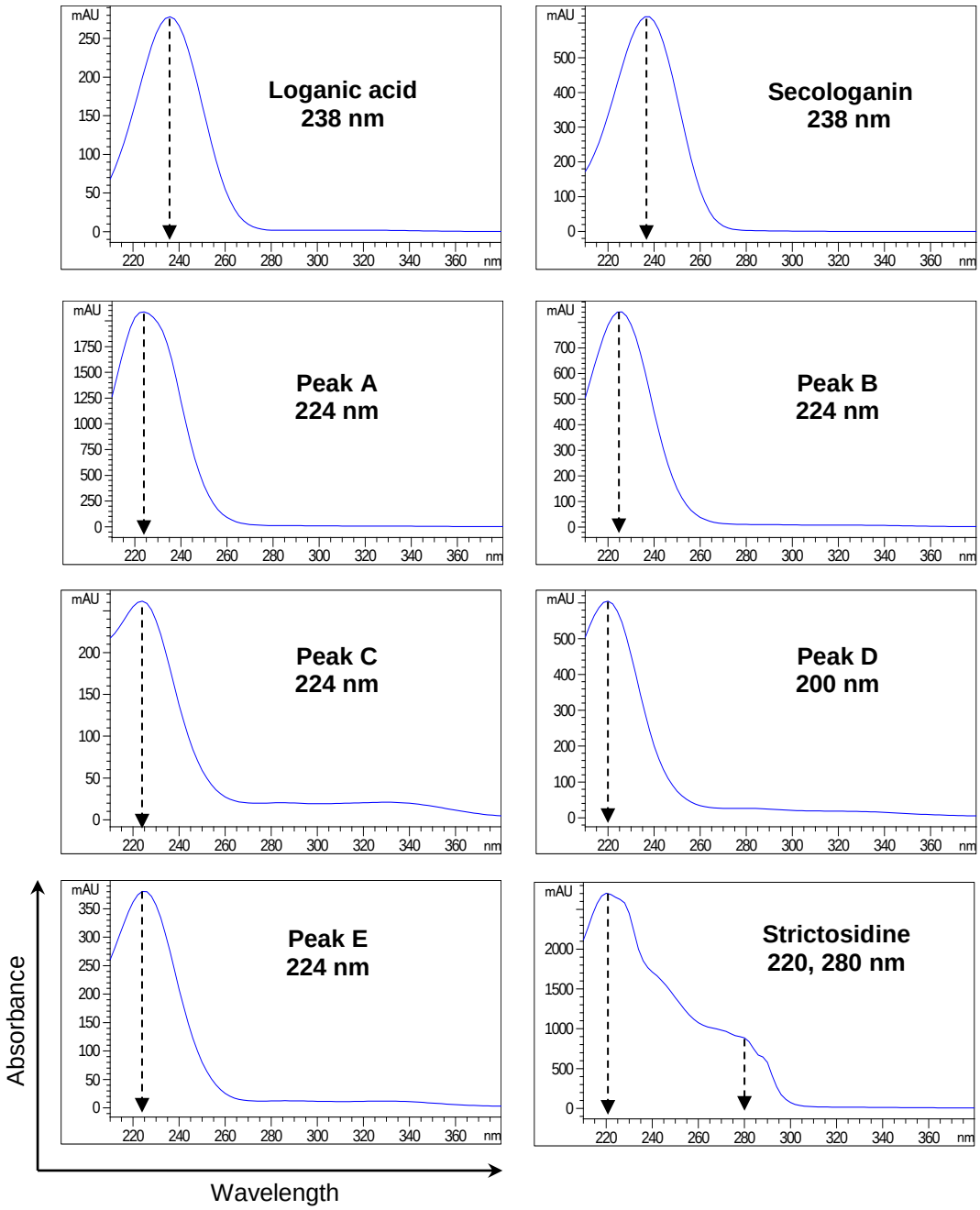


**Fig. 9** UV absorption spectra of standard compounds loganic acid, secologanin, strictosidine, and the new peaks (labelled as A, B, C, D, and E) detected geraniol-fed (1.62 mM) *Catharanthus roseus* cell culture (cell-line CRPP). X-axis is wavelength (nm) and Y-axis is absorbance (mAU).

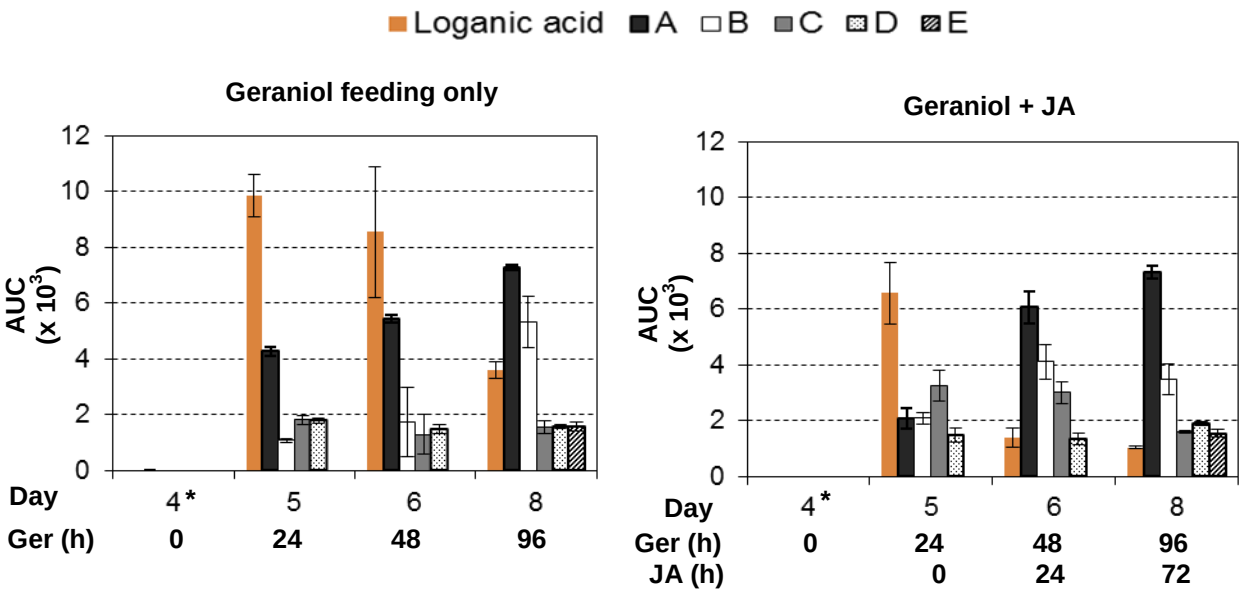


**Fig. 10** Relative levels of peak A, B, C, D, E, and loganic acid in *Catharanthus roseus* cell cultures (cell-line CRPP) fed with 1.62 mM geraniol (Ger) at day 4. Geraniol + JA is combination of Ger feeding (1.62 mM) at day 4 and jasmonic acid (JA) elicitation (102 µM) at day 5. Error bars represent standard deviations obtained from three replicates. AUC is area under the curve value determined by HPLC-Diode Array Detector (DAD). *Samples at day 4 are not analyzed.

elicitation, while peak E was only detected at 96 hours after geraniol feeding and it was not affected by JA treatment. The structures of these compounds remain to be determined using further spectroscopic analyses.

The samples of geraniol feeding and the control were analyzed using NMR spectroscopy. Several proton signals appeared in the spectra of the extracts of the treated cells which are not present in the control (**Fig. 11).** Based on the $^{1}$H- and 2D-NMR spectra (**Supplement 2 – 6**), the compounds might be analogues of geraniol due to the characteristic signals at $\delta$ 6.98 (*t*, *J* = 7.6 Hz), $\delta$ 1.87 (*s*), and $\delta$ 2.28 (*m*) which have correlation to carboxyl groups at C-8 (HMBC: $\delta$ 170). In addition, the doublets at $\delta$ 5.56 – $\delta$ 5.57 (*J* = 8.0 Hz) and multiplets at $\delta$ 3.48 – $\delta$ 3.51 indicates the signals of glycosides. However, the complete structure of the compounds are not yet clear and cannot be deduced from the spectrum because of the complexity of the mixture. Further work should be done to isolate the compounds by preparative HPLC. After recovery of the pure compounds, structure elucidation by NMR- and MS-spectroscopy should lead to the complete structures.

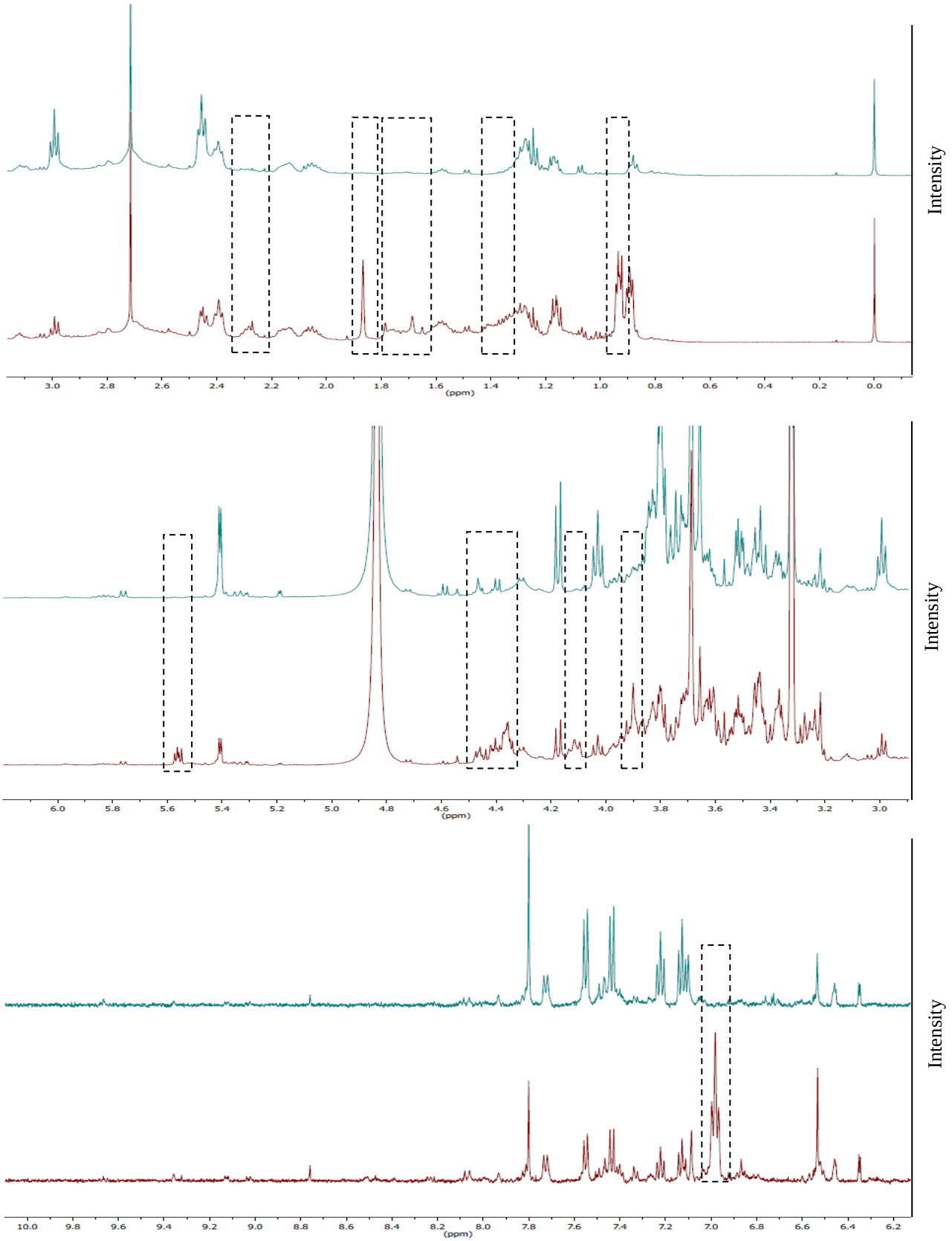


**Fig. 11** $^{1}$H-NMR spectra of geraniol-fed (brown) and control (blue) cell suspension cultures of *Catharanthus roseus*. Several new $^{1}$H signals present in the geraniol-fed cell (brown).

## Conclusion

The *C. roseus* CRPP cell line can sustain a high concentration of geraniol without strong adverse effects on biomass accumulation, thus it is a useful system to study the geraniol and iridoid pathway, and the effects of plant signaling molecules such as JA. Feeding the final concentration of 1.62 mM geraniol resulted in high accumulation of loganic acid and some geraniol analogues, but might repress TIA production. Jasmonic acid elicitation increases all TIA compounds detected in the cell suspension culture. A combination of geraniol feeding with subsequent JA elicitation increased the strictosidine level, but not the down-stream TIA if compared to the only elicitation treatment. Further studies on the flow of carbon in different metabolic pathways need to be established as in the current study the measured values only minus catabolism. Moreover, different ranges of geraniol concentrations need to be explored for further optimization of TIA production. In addition, the optimal timing of feeding and elicitation must be established in follow-up studies.

## Acknowledgements

The authors thank the Ministry of Education Malaysia and University of Malaya, Malaysia for the financial support of Mohd Zuwairi Saiman. This research was funded by the IBOS-ACTS program as coordinated by NWO.

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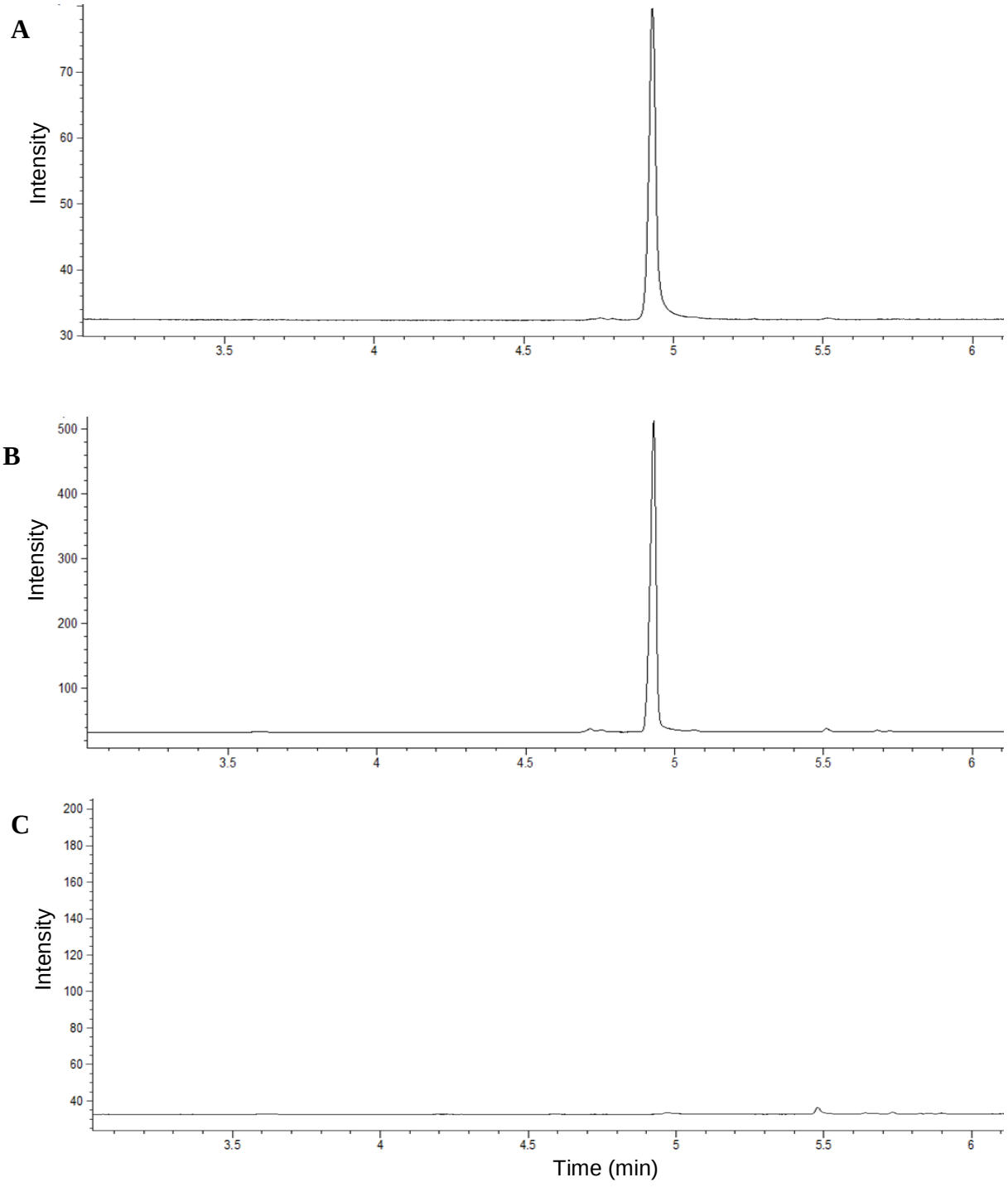
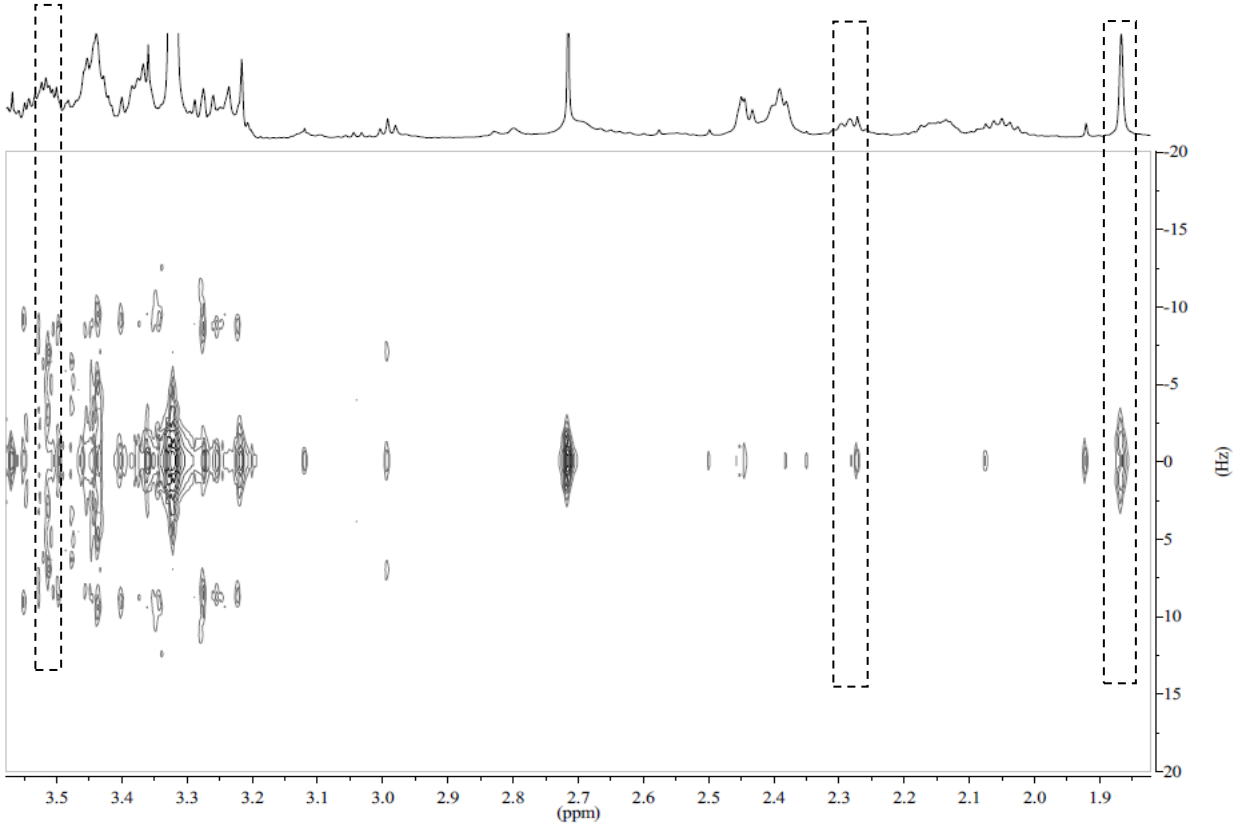
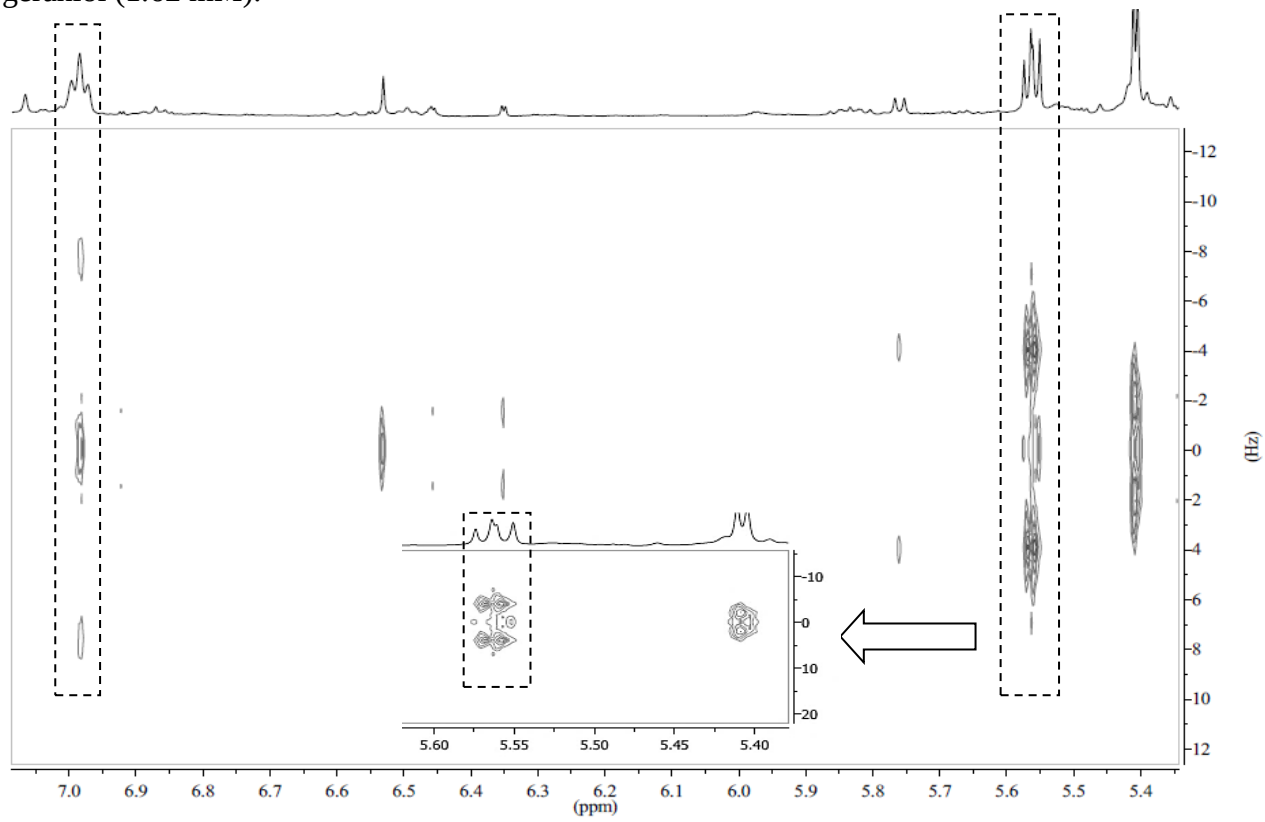
**Supplement 1** Chromatograms of GC-Flame Ionization Detector (FID) of geraniol standard compound (A), and liquid medium of *Catharanthus roseus* cell suspensions at 0 hour (B) and 24 hours (C) after geraniol feeding (1.62 mM), respectively.



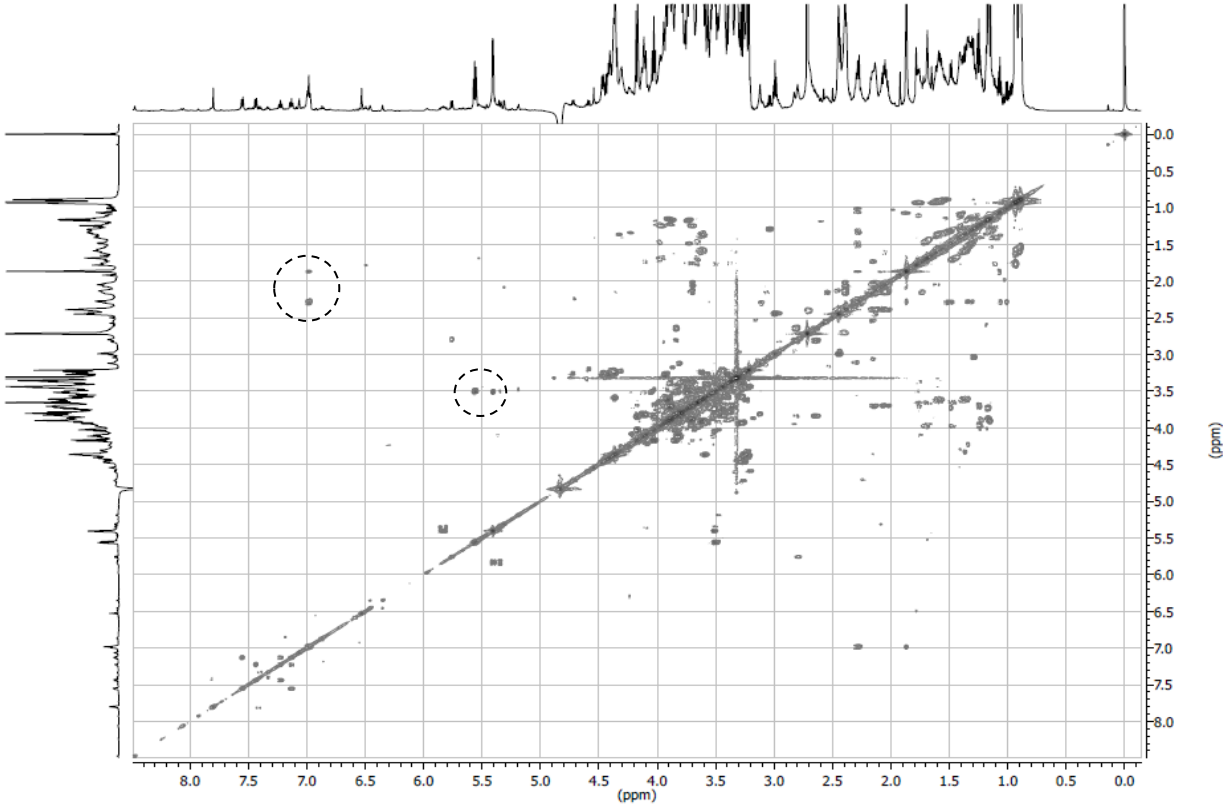
**Supplement 2** *J*-resolved $^{1}$H-NMR ($\delta$ 1.85 – $\delta$ 3.55) of *Catharanthus roseus* cells fed with geraniol (1.62 mM)



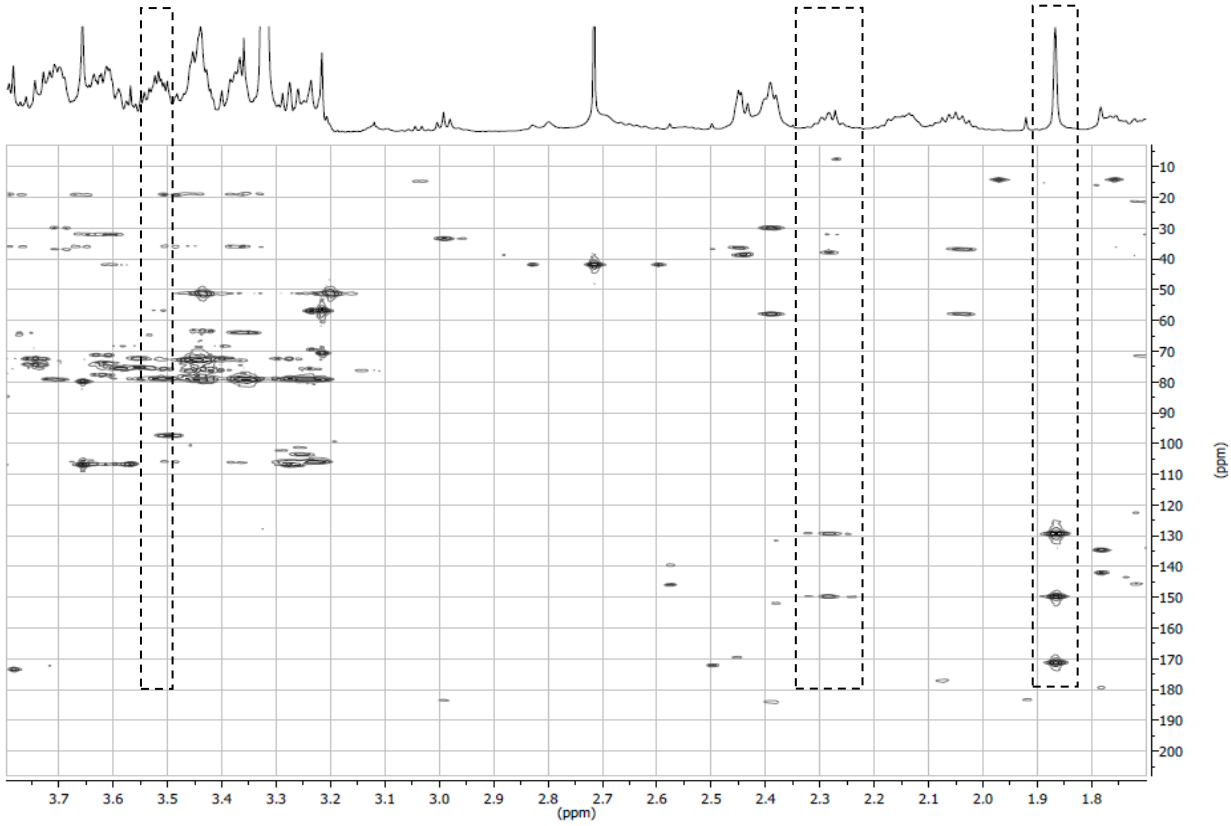

**Supplement 3** *J*-resolved $^{1}$H-NMR ($\delta$ 5.35 – $\delta$ 7.05) of *Catharanthus roseus* cells fed with geraniol (1.62 mM).



**Supplement 4** COSY spectrum ($\delta$ 0.00 – $\delta$ 8.50) of *Catharanthus roseus* cells fed with geraniol (1.62 mM).




**Supplement 5** HMBC spectrum ($\delta$ 1.70 – $\delta$ 3.80) of *Catharanthus roseus* cells fed with geraniol (1.62 mM).



**Supplement 6** HMBC spectrum ($\delta$ 3.20 – $\delta$ 7.30) of *Catharanthus roseus* cells fed with geraniol (1.62 mM).