Chapter 3

Metabolic alterations and distribution of five-carbon terpenoid precursors in jasmonic acid-elicited *Catharanthus roseus* cell suspension cultures

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Abstract

Catharanthus roseus produces an important group of secondary metabolites known as terpenoid indole alkaloid (TIA). In *C. roseus* cell cultures, the pathway of the terpenoid moiety is considered as the limiting factor in TIA production. The pathway of terpenoidal precursors in TIA is strongly linked with other terpenoid pathways, which suggests that TIA production might be limited by competition for the isopentenyl precursors. This raises the question whether the stimulation of TIA production by certain signal molecules is due to a redistribution of precursors between the associated terpenoid pathways and/or to a total increase of precursor availability. To investigate the effect of a TIA-increasing signal molecule, the cell suspension cultures of *C. roseus* were elicited with jasmonic acid (JA) and the metabolic changes of different terpenoid pathways were evaluated targeting on TIA (monoterpenoid; C10), carotenoid (tetraterpenoid; C40), and phytosterol (triterpenoid; C30). The results of HPLC-DAD or GC-FID analyses showed that TIA and carotenoid levels almost doubled upon JA elicitation, while phytosterol levels remained constant if compared to the control. This indicates that only these two pathways benefit from an increased flow in the MEP pathway, and that export of IPP::DMAPP intermediates to the cytosolic terpenoid routes like the phytosterol is minimal. Furthermore, the metabolites profiles observed by ^{1}H-NMR spectroscopy and analyzed by multivariate data analysis showed a discrimination of JA-elicited and control cells between 24 – 72 hours after treatments. In JA-elicited cells, the levels of strictosidine and organic acids in the TCA cycle, i.e. succinic acid, fumaric acid, and malic acid were increased, whereas sugars levels were decreased. This study portrays metabolic alterations upon JA elicitation in different biosynthetic pathways, which provides a knowledge platform for developing strategies to engineer fluxes in a complex biosynthetic network in order to obtain high TIA-producing *C. roseus* cell lines.

Introduction

Catharanthus roseus plants produce a diverse array of secondary metabolites including terpenoid indole alkaloids (TIA), which are a valuable and unique source of the pharmaceutically important anticancer compounds vincristine and vinblastine. The other important TIA produced by *C. roseus* are ajmalicine and serpentine having antihypertensive activity (Moreno et al. 1995; van der Heijden et al. 2004).

Plants require a long cultivation period and produce only low amounts of secondary metabolites. Therefore, cell culture systems are explored as an alternative source of valuable plant metabolites. Despite some successful examples (Verpoorte et al. 2002), the production

of secondary metabolites in most plant cell cultures is relatively low or sometimes zero. In the case of *C. roseus*, bisindole alkaloids such as vincristine and vinblastine are not produced in cell cultures due to the lack of one of the biosynthetic precursor, vindoline. Nevertheless, semi-synthesis of bisindole alkaloids is possible by coupling vindoline and catharanthine, thus optimizing cell cultures to produce the individual precursors is an interesting target. Although ajmalicine and serpentine accumulate in *C. roseus* cell cultures, the productivity is too low compared to the intact plants for a cost-competitive mass production (Zhao and Verpoorte 2007).

Several strategies can be applied to improve product yields in plant cell cultures. One of the techniques is to induce the biosynthesis of secondary metabolites by elicitation. Elicitation strategies using exogenous jasmonic acid (JA) or its volatile methyl ester, methyl jasmonic (MeJA), often result in elevated levels of certain secondary metabolites. In the elicitation process, jasmonates play an important role as regulatory signals to induce *de novo* transcription and translation leading to the induction of secondary metabolite biosynthesis in plant cell cultures (Gundlach et al. 1992; Memelink et al. 2001; Zhao and Verpoorte 2007). Exogenous application of jasmonates to *C. roseus* cell cultures (El-Sayed and Verpoorte 2002; Lee-Parsons and Royce 2006; Vázquez-Flota et al. 2009), hairy roots (Rijhwani and Shanks 1998; Vázquez-Flota et al. 2009), shoot cultures (Vázquez-Flota et al. 2009) and seedlings (El-Sayed and Verpoorte 2004) increased the production of TIA caused by an elevated expression of a set of biosynthesis related genes (Memelink et al. 2001). Combination of transcript and metabolic profiling of jasmonate elicited *C. roseus* cell cultures yielded a collection of known and previously undescribed transcript tags and metabolites associated with TIA (Rischer et al. 2006). Therefore, JA treatment is a useful biochemical tool to stimulate the production and to study the regulation of TIA biosynthesis.

Terpenoid indole alkaloids are derived from strictosidine which is formed by the coupling of the indole tryptamine and the iridoid secologanin, in which the latter are considered as the limiting factor of TIA biosynthesis in *C. roseus* cell cultures (Moreno et al. 1993; Whitmer et al. 2002). One of the limitations could be the competition at the level of IPP::DMAPP, the precursors of different terpenoid groups such as monoterpenoids (C10), triterpenoids (C30) or tetraterpenoids (C40). Furthermore, several studies showed an interaction between the precursor pools in cytosolic mevalonate and plastidial MEP pathway (Schuhr et al. 2003; Hemmerlin et al. 2003; Laule et al. 2003).

In this study, the effect of jasmonic acid elicitation on different terpenoid pathways, i.e. monoterpenoids (TIA), triterpenoids (phytosterols), and tetraterpenoids (carotenoids), and

the distribution of five-carbon precursors (C5) into representative terpenoid groups were evaluated. The transient accumulation of TIA, carotenoid, and phytosterol were analyzed by high performance liquid chromatography-diode array detector (HPLC-DAD) or gas chromatography-flame ionization detector (GC-FID). In addition, the metabolomic changes between control and treated samples were analyzed with our NMR-based metabolomics platform.

Materials and Methods

Cell culture materials

The *Catharanthus roseus* cell suspension culture (CRPP cell line) was obtained from the Natural Products Laboratory, Institute of Biology, Leiden University, The Netherlands. The cell suspension culture was subcultured every three weeks by diluting the cultures with fresh medium (1:1) consisting of B5 medium (Gamborg et al. 1968) supplemented with 1.86 mg/l NAA and 20 g/l glucose. The medium was adjusted to pH 5.8 before autoclaving at 121 ^{o}C for 20 min. The cultures were maintained in 250 ml shake flasks closed with T-32 silicon foam stoppers (Shin Etsu, Tokyo, Japan) and placed on a gyratory shaker (110 rpm) at 25 ^{o}C in continuous light (10.8 – 27 µE/m^2/sec). For the experiment, 20 ml (approx. 4 g fresh weight) of a two-week-old cell suspension culture was inoculated into 50 ml culture medium in the same cultivation conditions for 5 days prior to elicitation.

Elicitation

The stock solution of jasmonic acid (10 mg/ml) was prepared by dissolving jasmonic acid (Sigma-Aldrich Chemie, Steinheim, Germany) in 2 ml ethanol and diluted with sterile water to acquire the total volume of 10 ml. The solution was mixed and filter sterilized through 0.22 µm Millex^{TM} filter (Millipore, Bedford, MA, USA). At the fifth day of culture, 50 µl of jasmonic acid solution was added into the cell culture (70 ml) to achieve the final concentration of 34 µM (0.5 mg/flask). The same amount of control solution (20% v/v ethanol) was applied to the cell cultures. The treated and control cultures were performed in triplicate flasks and harvested at 0, 2, 6, 24, 48, and 72 hours after elicitation. The cells were filtered under reduced pressure, washed three times with deionized water, and lyophilized for 72 hours.

Analysis of terpenoid indole alkaloids, carotenoids, and sterols

Analysis of terpenoid indole alkaloids, carotenoids, and sterols were carried out according to the methods described by Saiman et al. (2014) (Chapter 2). Briefly, 100 mg of the freeze-dried cells were weighed and extracted for analyzing TIA and sterols, while 70 mg freeze-dried cells were extracted for carotenoids. Analyses of carotenoids, TIA and TIA precursors were performed using different HPLC-DAD methods, whereas the GC-FID system was used to analyze sterols. Calibration curves of the standard compounds were made for quantitative analyses. Results were presented as an average of the analyses of two separate flasks for several time-points (0, 6, 24, and 72 hours after treatment).

NMR measurement

NMR analysis was conducted according to the protocol of Kim et al. (2010). The freeze-dried cells (25 mg) from triplicate flasks of each treatments (JA and control) and time-points (0, 2, 6, 24, 48, 72 hours) were extracted with 1.2 ml of CD_3OD and 0.3 ml of KH_2PO_4 buffer in D_2O (pH 6.0, containing 0.01% w/w trimethylsilyl propanoic acid (TMSP) as internal standard). The mixture was vortexed for 10 sec, sonicated for 10 min, and centrifuged for 15 min (14,000 rpm). The supernatant was transferred into NMR tube for measurement of ^{1}H-NMR 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 TMSP at 0.0 ppm by using XWIN NMR version 3.5 (Bruker). The AMIX software (Bruker) was used to reduce the ^{1}H-NMR spectra to an ASCII file, with total intensity scaling. Bucketing or binning was performed and the spectral data were reduced to include regions of equal width (δ 0.04) corresponding to the region of δ 0.40 – 10.00. The regions of δ 4.75 – 4.90 and δ 3.30 – 3.35 were not included in the analysis because of the remaining signal of D_2O and CD_3OD, respectively. Multivariate data analysis was performed with the SIMCA-P+ software version 12.0 (Umetrics, Umeå, Sweden). The *t*-test was performed using IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL, USA) to determine statistical significance ($P < 0.05$) of the relative levels of metabolites.

Results and Discussion

Cell culture material

In the previous chapter, several *C. roseus* cell lines were analyzed for the accumulation of different terpenoid groups. The CRPP cell lines were found to have high productivity in terms of quantity and diversity of TIA and terpenoid compounds as compared to the other cell lines. Therefore, we used the CRPP cell line as a model to study the effect of jasmonic acid (JA) elicitation towards the accumulation of different terpenoid end-products derived from the mevalonate and MEP pathways including the distribution of five-carbon precursors (C5). Even though the sucrose supplemented CRPP cell line had a slightly higher TIA content, the glucose line was preferred for this study as we may pursue feeding with labeled glucose in later experiments to determine the fluxes of the carbons through the mevalonate route and the MEP pathway.

To estimate the best time-point for elicitation studies, the growth curve of the CRPP cell line was determined (**Fig 1**). Based on the cell growth curve, the elicitation experiment was performed at day 5, as the maximum biomass is reached and high metabolic rates can be achieved.

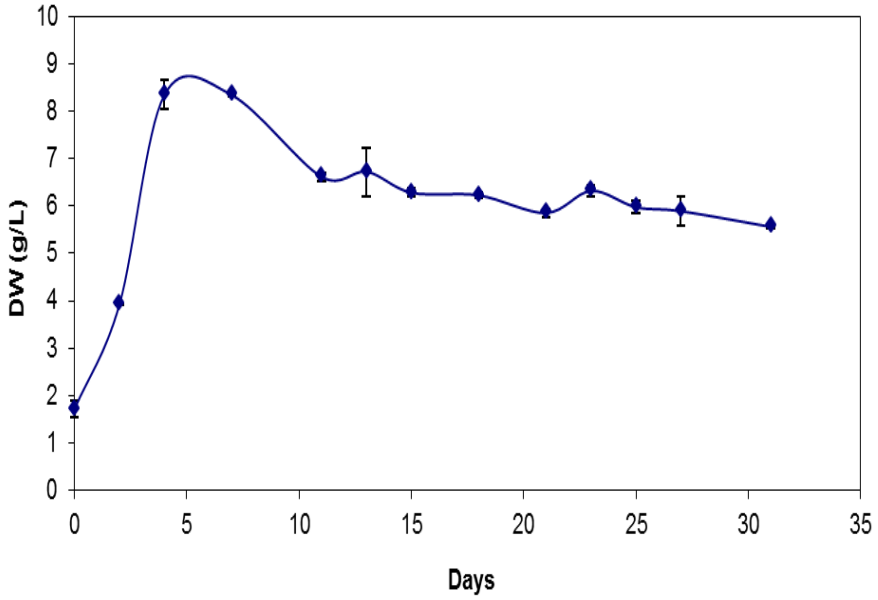


Fig. 1 The biomass accumulation of CRPP (glucose) cell line at different time-points after inoculating 20 ml of a three-week-old culture into 50 ml fresh medium. The cell dry weight (DW) was determined from duplicate flasks; error bars indicate the two values.

Analysis of terpenoid indole alkaloids and precursors

The analysis of iridoids showed only the presence of loganic acid, whereas loganin and secologanin, if present, were below detection limit. Neither tryptophan nor tryptamine was detected in the cell suspension culture. TIA analysis only showed strictosidine as major alkaloid and smaller amounts of serpentine, ajmalicine, and tabersonine. **Figure 2** shows the levels of loganic acid and TIAs at different time-points after jasmonic acid elicitation. Loganic acid, the iridoid precursor of TIA was twofold higher in the JA-elicited cells 6 hours after elicitation. Although loganic acid concentration was still higher compared to the control

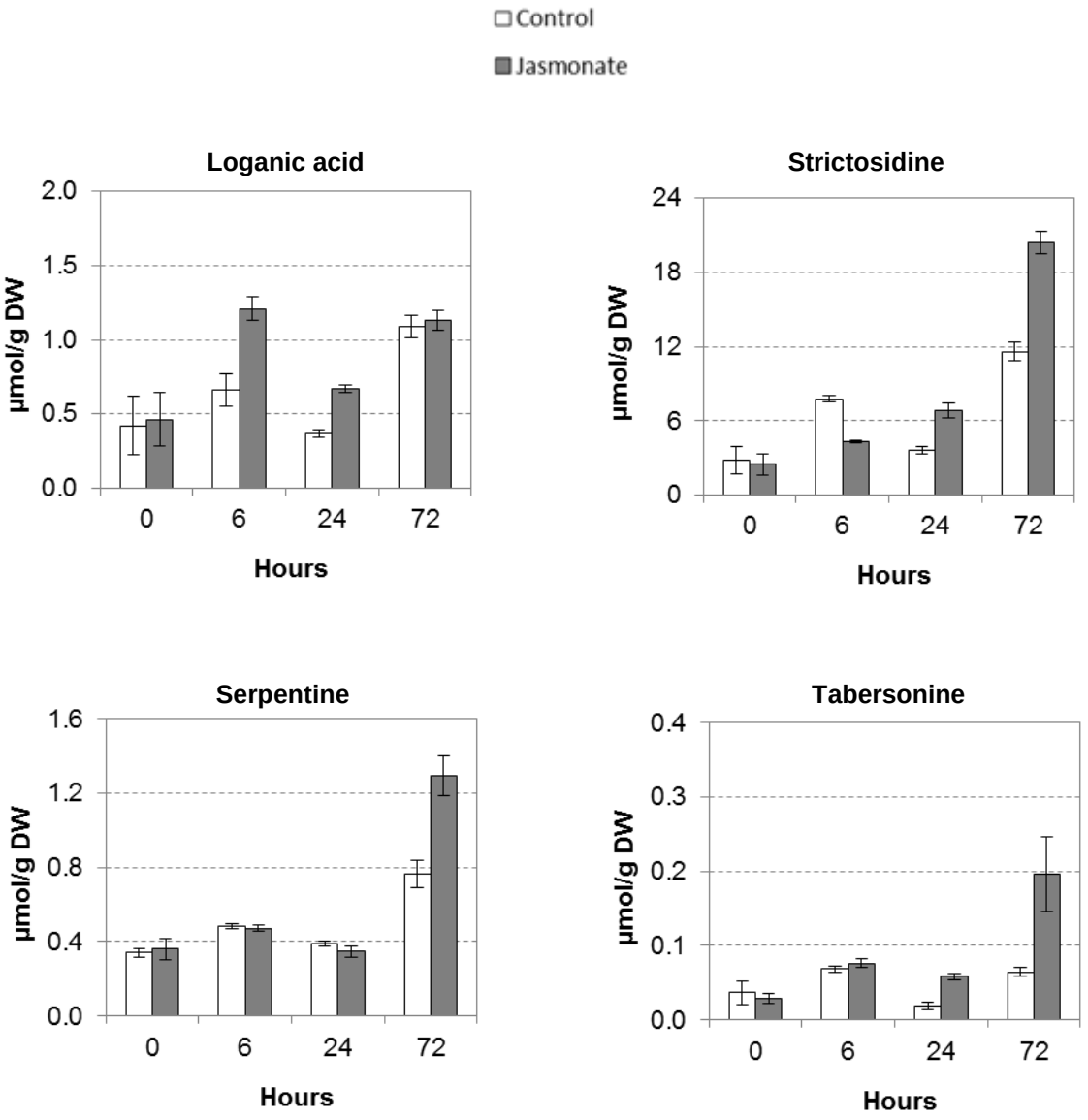


Fig. 2 Time course of loganic acid and terpenoid indole alkaloid production in CRPP cell line elicited with 34 µM jasmonic acid or control solution measured by HPLC-Diode Array Detector (DAD). Results are the mean of two replicates; error bars indicate the two values.

at 24 hours, the level was lower than at 6 hours JA elicitation. At the later time-points, loganic acid levels in both elicited and control cells were not different. The central intermediate of TIA, strictosidine, was about twofold increased at 24 and 72 hours after elicitation, while the serpentine level was only increased at the latter time-point. The tabersonine level was also increased (threefold) at 72 hours after elicitation. Overall, loganic acid concentration was increased between 6 – 24 hours after elicitation and subsequently the levels of strictosidine and the other down-stream TIAs gradually increased in time.

It is known that the increased levels of TIA upon jasmonate elicitation results from elevated expression of several genes encoding enzymes involved in TIA biosynthesis (Memelink et al. 2001). The jasmonate-responsive expression of a number of these biosynthesis genes is controlled by transcriptional regulators known as octadecanoid-derivative responsive *Catharanthus* AP2-domain (ORCA) proteins (Memelink et al. 2001). ORCA3 was shown to regulate the expression levels of *DXS*, *ASα*, *TDC*, *CPR*, *STR*, and *D4H* but not of *G10H* (or also known as *G8O*), *SGD*, and *DAT* (van der Fits and Memelink 2000). Another AP2/ERF-domain transcription factor called ORCA2 was not fully characterized but it was found to affect the expression of *STR* and *TDC* (Menke et al. 1999). ORCA2 and ORCA3 have been suggested to regulate different but overlapping sets of genes (Memelink et al. 2001). A study by Hasnain et al. (2010) showed that *ASα*, *TDC*, *SLS*, *STR*, and *D4H* genes were upregulated by both ORCA2 and ORCA3.

It should be noted that the composition of strictosidine, serpentine, and tabersonine in the control CRPP cell line at the 72 hour time-point was 93.3%, 6.1%, and 0.5% respectively, whereas after elicitation, those compounds were all increased but the relative composition was not really changed with strictosidine (93%), serpentine (6%), and tabersonine (1%). This shows that the flux through the pathway is increased, but within the TIA biosynthetic network the enzyme activities remain the same for the enzymes involved in the biosynthesis of serpentine and tabersonine, and apparently the activities are not fully used. This fits with the observations of Whitmer et al. (2002) who showed that by feeding TIA precursors the TIA network has a large overcapacity that under normal condition is not used.

Analysis of carotenoids

Carotenoids belong to the tetraterpenoids (C40) group and are derived from GGPP formed from the C5 precursors of the MEP pathway localized in the plastids. The key step of carotenoid biosynthesis is the formation of lycopene which is derived from phytoene after condensation of two GGPP molecules. Lycopene is a branch point for the production of two

cyclic carotenoids, *α*-carotene and *β*-carotene. Lutein derives from *α*-carotene, while *β*-carotene produces oxygenated derivatives of zeaxanthin, violaxanthin, and neoxanthin (Namitha and Negi 2010).

An increase level of carotenoids can be observed at 72 hours after JA elicitation in the *C. roseus* cell suspension culture (**Fig. 3**). At that time-point, the concentrations of all measured carotenoids in this study were about twofold higher in the JA-treated cells compared to the control. In addition, the levels of chlorophyll a and b were also increased after 72 hours in JA-treated cells (**Fig 4**). The composition of the mixture of carotenoids in the *C. roseus* cell culture did not alter after JA treatment; *β*-carotene stands for the largest part of the total carotenoids followed by lutein, neoxanthin, and violaxanthin.

Previous studies showed that jasmonate regulates the biosynthesis of carotenoids (Saniewski and Czapski 1983; Pérez et al. 1993; Rudell and Mattheis 2002; Liu et al. 2012). Jasmonate elicitation increased the expression level of a number of biosynthesis genes including *DXS* (van der Fits and Memelink 2000), encoding an enzyme of upstream part of the MEP pathway. Overexpressing the *DXS* gene in transgenic *Escherichia coli* resulted in an increased accumulation of the carotenoids lycopene (Harker and Bramley 1999; Matthews and Wurtzel 2000), *β*-carotene (Albrecht et la. 1999) or zeaxanthin (Albrecht et la. 1999; Matthews and Wurtzel 2000). Furthermore, the *GGPPS* transcript levels in *Taxus canadensis* cell suspension cultures (Hefner et al. 1998) and *Corylus avellana* leaves (Wang et al. 2010) were up-regulated after MeJA treatment, even though no significant change in *GGPPS* expression was observed in *C. roseus* cell suspension cultures (Thabet et al. 2012). Thabet et al. (2012) speculated that there are several homologues of GGPPS in *C. roseus* and only some of them may be induced by MeJA as exemplified in tomato (*Lycopersicon esculentum*), in which *LeGGPPS1* was induced by jasmonate acid but not the *LeGGPPS2* (Ament et al. 2006).

Carotenoids are essential in photosynthesis to absorb light energy and protect chlorophylls from photo damage (Namitha and Negi 2010). Pérez et al. 1993 showed that MeJA stimulates chlorophyll degradation in ‘Golden Delicious’ apple peel and chlorophyll a/b ratio decreases with increasing MJ exposure. In contrast, Rudell and Mattheis (2002) found a significant increase of chlorophyll b, while the level of chlorophyll a was relatively stable after MeJA treatment in ‘Fuji’ apples. Rudell and Mattheis (2002) suggested that the different results are probably due to the light treatment which was not applied by Pérez et al. (1993). In agreement with Rudell and Mattheis (2002), a notable increase of both chlorophylls a and b were observed at 72 hours after JA elicitation in our *C. roseus* cell

suspension cultures under light treatment. The chlorophyll a and b were increased in our study, this maybe the result of elevated activity of light harvesting connected with the increased level of carotenoids at that time-point (**Fig. 3** and **4)**.

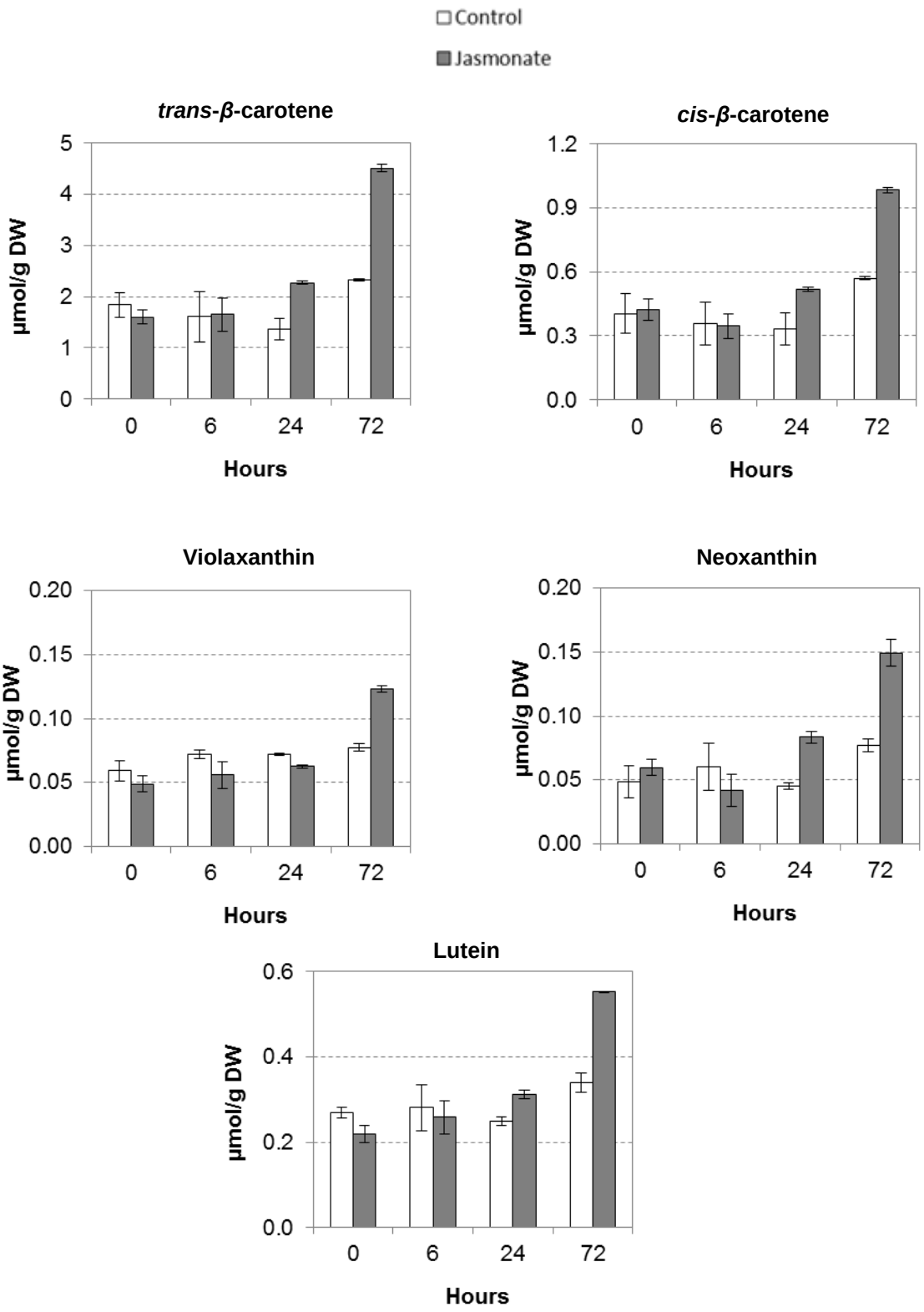


Fig. 3 Time course of carotenoid production in CRPP cell line elicited with 34 µM jasmonic acid or control solution measured by HPLC-Diode Array Detector (DAD). Results are the mean of two replicates; error bars indicate the two values.

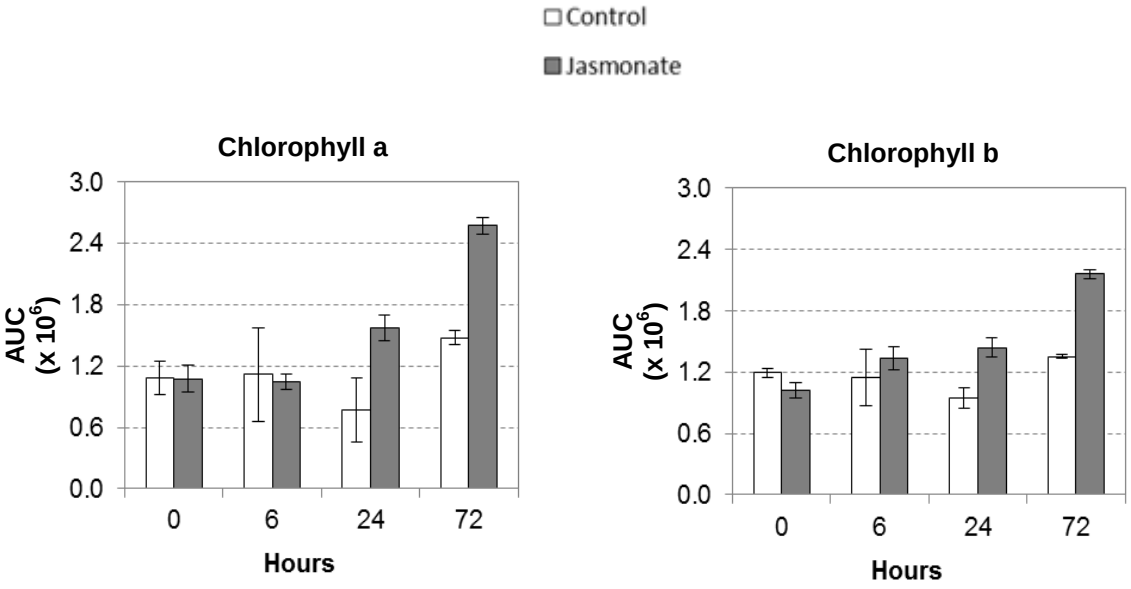


Fig. 4 Chlorophyll levels in CRPP cell line elicited with 34 µM jasmonic acid or control solution measured by HPLC-Diode Array Detector (DAD). Results are the mean of two replicates; error bars indicate the two values. AUC is area under the curve value.

Analysis of phytosterol

The levels of campesterol, *β*-sitosterol, and stigmasterol were not relatively different between JA-elicited and control cells (**Fig. 5)**. This indicates that JA elicitation at the concentration applied did not alter the biosynthesis of phytosterols. Studies in *Glycyrrhiza glabra* cell cultures (Hayashi et al. 2003) and the adventitious root cultures of *Panax ginseng* (Lee et al. 2004) showed that MeJA treatment did not induce the transcript level of cycloartenol synthase, the enzyme responsible for the first step in sterol biosynthesis. However, several studies showed contradictory results on phytosterols accumulation after MeJA elicitation in different plants or plant cell cultures. For example, phytosterols production was enhanced in *Lemna paucicostata* cultured plants (Suh et al. 2013) and *Capsicum annuum* cell culture (Sabater-Jara et al. 2010), decreased in *Centella asiatica* and *Ruscus aculeatus* cultured plants (Mangas et al. 2006), and remained constant in *Galphimia glauca* cultured plants (Mangas et al. 2006) and *Glycyrrhiza glabra* cell cultures (Hayashi et al. 2003). The different results may indicate the existence of different control mechanisms among the plant species.

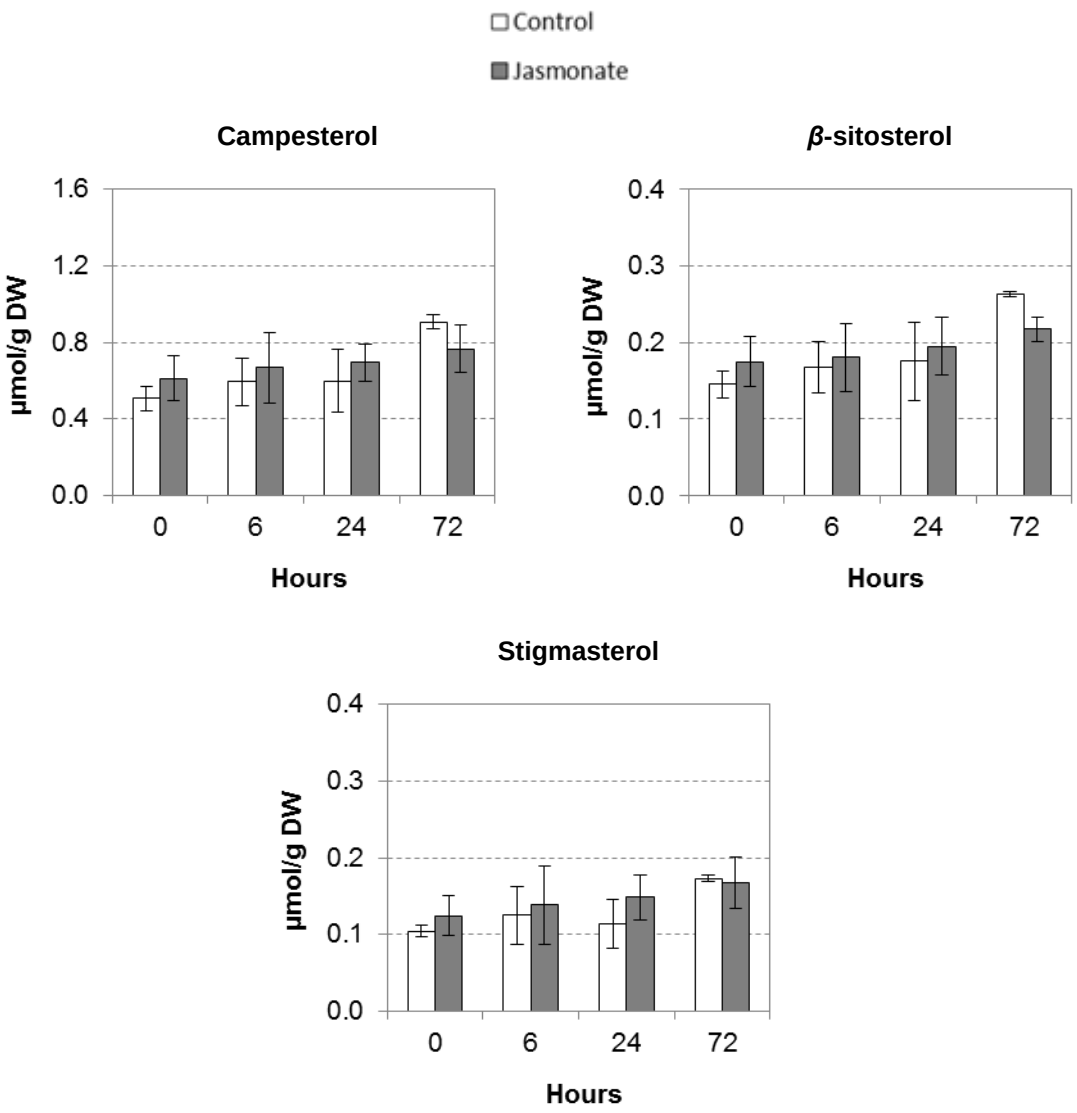


Fig. 5 Time course of sterols production in CRPP cell line elicited with 34 µM jasmonic acid or control solution measured by GC-Flame Ionization Detector (FID). Results are the mean of two replicates; error bars indicate the two values.

Total accumulation and C5 distribution

The effect of JA elicitation in terms of total accumulation of the various terpenoid groups and the distribution of five-carbon precursors (C5) was evaluated. In terms of total accumulation of the terpenoid groups, monoterpenoids consisting of loganic acid and several TIAs were highly accumulated during the growth time-course and after elicitation compared to carotenoids and sterols (**Fig. 6a).** The major contribution (82%) to the total composition of monoterpenoid accumulation comes from strictosidine, which indicates that the flux towards strictosidine is highly active in this specific *C. roseus* cell line. Total phytosterols content was relatively constant, whereas total carotenoids level was increased at 24 and 72 hours after JA

elicitation. These results suggest a relationship between TIA and carotenoid pathways induction as can be explained by up-regulation of several genes encoding enzymes in the MEP, TIA, and carotenoid pathways upon jasmonate elicitation (Thabet et al. 2012; van der Fits and Memelink 2000).

As regards to the distribution of C5 precursors, the level of C5 units increased in both carotenoids and TIA after 24 and 72 hours of elicitation, whereas phytosterols did not change compared to the control (**Fig. 6b**). This shows that upon jasmonate elicitation, the carbon flow is activated towards the MEP pathway rather than the MVA pathway leading to phytosterols, and that export of IPP::DMAPP intermediates to the cytosolic terpenoid routes like the phytosterol is minimal. At 24 hours after elicitation, the level of the C5 unit in TIAs was 1.8-fold higher in elicited cells (15.86 µmol/g DW) compared to the control (8.80 umol/g DW), whereas in carotenoids a 1.6-fold increase of C5 units was observed in the elicited cells (25.97 umol/g DW) compared to the control (16.45 umol/g DW). Subsequently, after 72

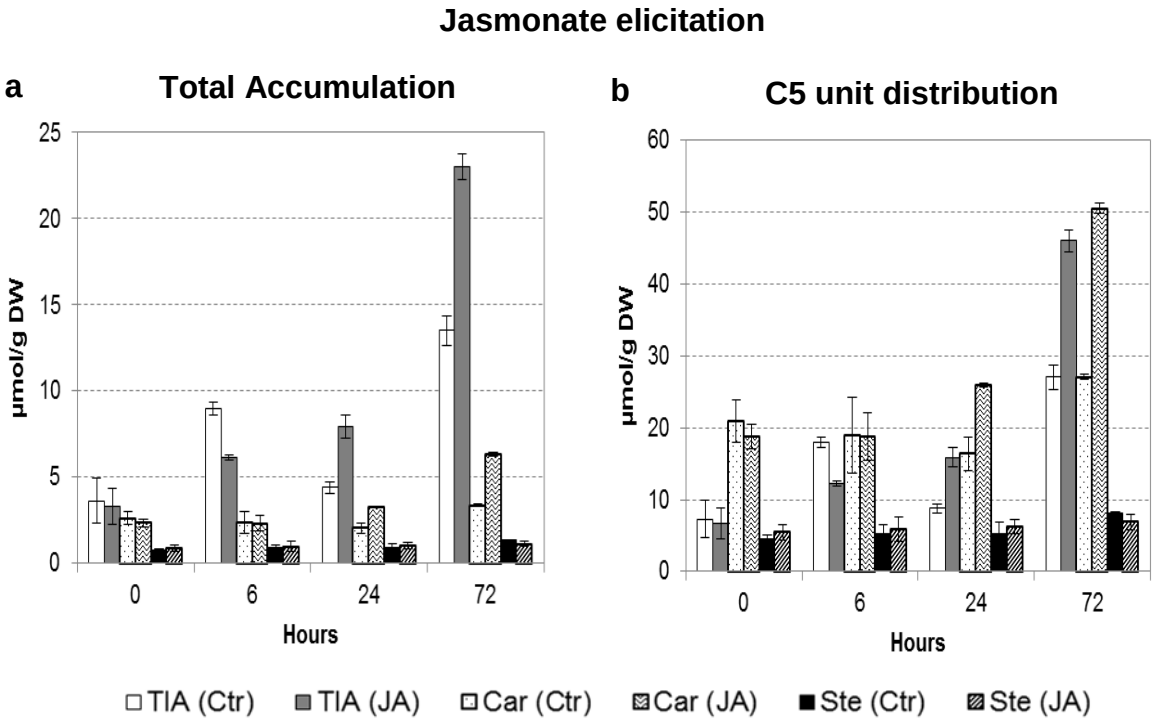


Fig. 6 Total accumulation (a) of terpenoid indole alkaloids, carotenoids, and sterol and the C5 precursor distribution (b) after jasmonic acid elicitation (34 µM) in the CRPP cell line. Results are the mean of two replicates; error bars indicate the two values.

hours of elicitation, the distribution of the C5 unit to these pathways was almost doubled and interestingly between and within these pathways the ratios of the compounds more or less remain the same as in the control. Pointing at a regulation at the very beginning of the MEP pathway producing more C5 units which are then, by the existing biochemical machinery converted to the various products resulting in similar ratios of these as in control cells. The results suggest that both carotenoid and TIA pathways benefit from an increased carbon flow in the MEP pathway, but that elicitation does not really change the carbon flux distribution between the two pathways. TIA production might be further increased by changing the C5 distribution into these pathways.

Metabolite profiles using NMR

The extracts of *C. roseus* cell suspension cultures harvested at different time-points after JA treatment and controls were subjected to ^{1}H-NMR analysis. **Fig 7** shows the comparison of ^{1}H-NMR spectra of JA-elicited and control samples after 72 hours treatment. The amino acids isoleucine, leucine, valine, and alanine were identified. Organic acids like acetic acid, succinic acid, malic acid, fumaric acid, and formic acid were also found. In the carbohydrate region, signals of anomeric protons of β-glucose, α-glucose, and sucrose were detected. The aromatic part of the ^{1}H-NMR spectra showed signals of indole moiety (twin triplets and doublets around δ 7.00 – δ 7.50) and the presence of signals at δ 7.07 (*t*, J = 7.5 Hz, H-10), δ 7.16 (*t*, J = 7.5 Hz, H-11), δ 7.37 (*d*, J = 8.0 Hz, H-12), δ 7.49 (*d*, J = 8.0 Hz, H-9), δ 7.80 (*s*, H-17), and δ 5.80 (*d*, J = 8.5 Hz, H-1') represent characteristic signals of strictosidine. The other compound detected is loganic acid by the presence of signals at δ 1.08 (*d*, J = 6.7 Hz, H-10), δ 4.68 (*d*, J = 8.0 Hz, H-7), δ 5.27 (*d*, J = 3.5 Hz, H-1), δ 7.08 (*s*, H-3). The assignments of ^{1}H-NMR signals were performed by comparing the reference spectra of our in-house database and the results of previous reports (Kim et al. 2006, 2010; Mustafa et al. 2009; Saiman et al. 2012).

The NMR method developed in our group has been successfully applied for metabolite analysis in plant extracts (Kim et al. 2010). It is a robust method for metabolic analysis particularly in terms of reproducibility. NMR methods offer some advantages compared to conventional chromatography or MS methods. The range of compounds is less limited by their polarity and specific chemical features such as chromophores, which means that a broader range of metabolites can be detected by NMR. The NMR extraction method is simple and fast. The solvent used can be adjusted for analysis of a particular target group. However, NMR detection may be less suitable for compounds present at relatively low levels

using the usual short time of sample analysis. Nevertheless, this limitation can be improved considerably by increasing the number of scans or using higher field strength. In addition, the application of cryoprobes and microprobes has greatly contributed to increase the NMR sensitivity (Kim et al. 2006, 2010). Two-dimensional NMR analysis can be applied to confirm the presence of a compound in the extract as well as to overcome in part the problem of overlapping signals.

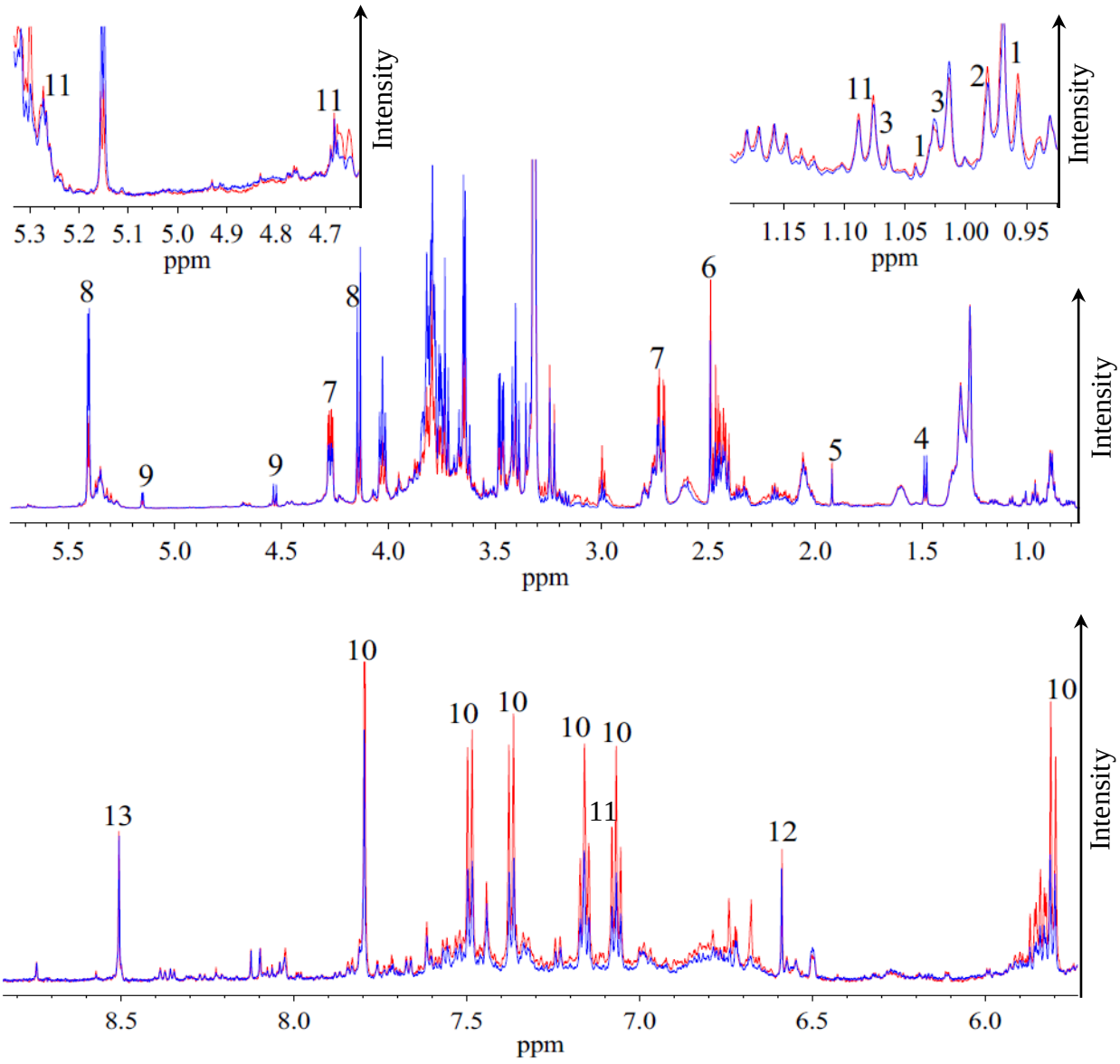


Fig. 7 ^{1}H-NMR spectra of jasmonate-elicited (red) and control (blue) cell suspension cultures of *Catharanthus roseus* at 72 hours. 1: isoleucine, 2: leucine, 3: valine, 4: alanine, 5: acetic acid, 6: succinic acid, 7: malic acid, 8: sucrose (fructose moiety: δ 4.14, glucose moiety: δ 5.41), 9: glucose (β-glucose: δ 4.53, α-glucose: δ 5.15), 10: strictosidine, 11: loganic acid, 12: fumaric acid, 13: formic acid.

Multivariate data analysis of NMR data

To analyze the changes in the metabolic profiles between JA-elicited and control cells of *C. roseus*, chemometric methods were applied. Principal component analysis (PCA) is the most common method used to analyze data that arises from more than one variable. PCA is an unsupervised clustering method requiring no knowledge of the data set, which is used to reduce the dimensionality of multivariate data and displays systemic variation between the samples in an unbiased way (Eriksson et al. 2001, 2006). In this study, PCA was applied to the bucketed data of ^{1}H-NMR spectra. The chemical shifts (ppm) were used as variables and the samples served as observations in the data matrix. The data was mean-centered and variables were scaled to the Pareto method. The results are presented in a graphical form known as score plot, which differentiates samples based on the variables. The loading plot is used to find variables, which contribute to separation of the samples.

Figure 8 presents the PCA score plot of the samples. The plot shows that the samples were separated due to the growth cycle, in which the older samples moved to the negative part of PC1. This indicates that the metabolites profiles change with the developmental status of the cells. The PCA did not exhibit a clear distinction at 0 – 6 hours after elicitation but a separation of the elicited and control samples was observed beyond 24 hours after elicitation. PCA is an unbiased method which shows maximum variation within the samples. If the biological variation among the replicates is larger than between the groups, a clear separation is not observed. This was in fact found here for the early time-point samples and as experienced by Ali et al. (2012) in infected and control samples of grapevine at different time intervals. An effect of the developmental stage on the metabolome was also found in our *C. roseus* cell-line A12A2 when studying the time-effect associated to salicylic acid elicitation (Mustafa et al. 2009).

To extract information about specific variables, a supervised multivariate analysis is needed. Thus, partial least squares-discriminant analysis (PLS-DA) was applied to the same bucketed ^{1}H-NMR spectra. In addition to the X-matrix of NMR data, two classes (JA-treated and control cell) were assigned for the Y-matrix in PLS-DA. The variables were mean-centered and scaled to unit variance. However, PLS-DA was unable to give any significant model that fitted to the data. It was decided to separate the samples into two groups; 0 – 6 hours and 24 – 72 hours since the earlier result in PCA shows such separation between JA elicitation and control samples beyond 24 hours of elicitation. As expected, the PLS-DA of the samples at 0 – 6 hours time intervals did not deliver any fit component model, which

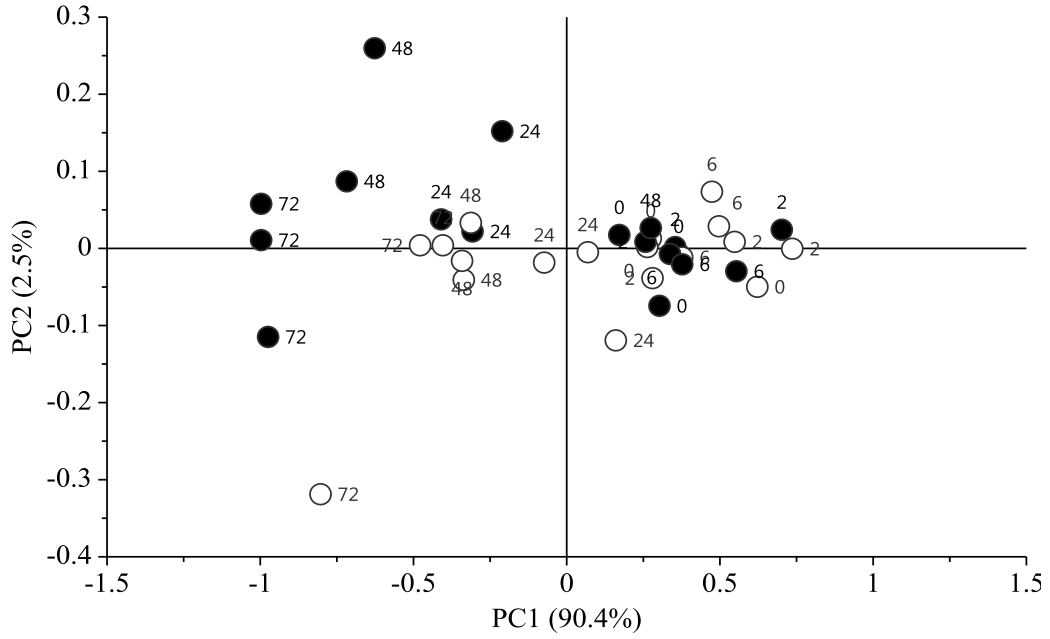


Fig. 8 Score plot of principal component analysis (PCA) of jasmonic acid-elicited (●) and control (○) samples of the CRPP cell line measured by ^{1}H-NMR. The numbers in the score plot are harvesting time (hour) after treatments.

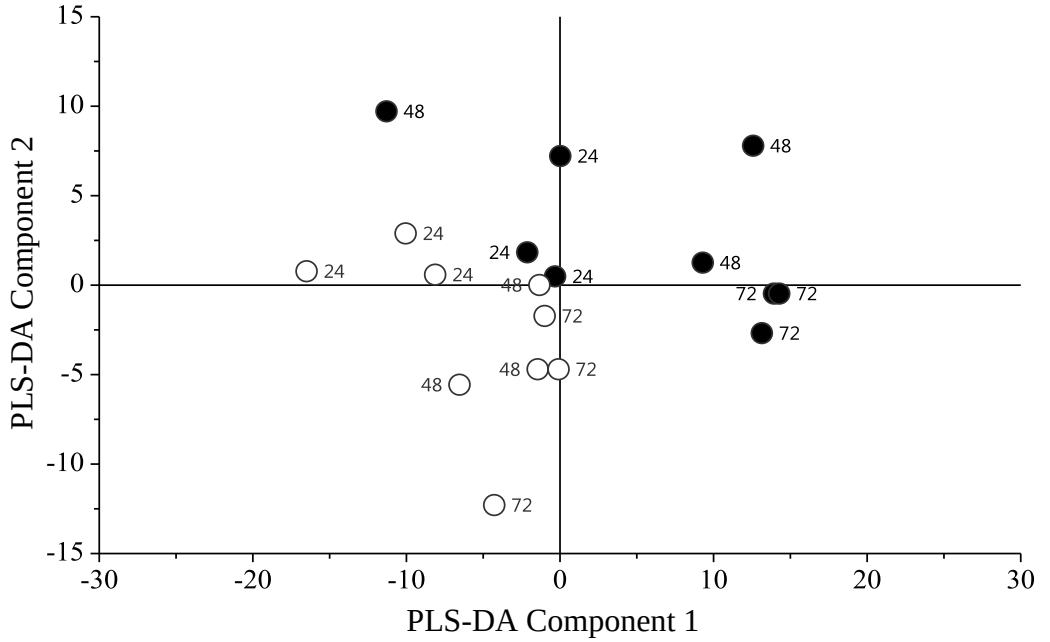


Fig. 9 Score plot of partial least squares-discriminant analysis (PLS-DA) of jasmonic acid-elicited (●) and control (○) samples of the CRPP cell line measured by ^{1}H-NMR. The numbers in the score plot are harvesting time (hour) after treatments.

indicates no significant separation among the samples. Instead, the samples at 24 – 72 hour time-points gave five component models with R2 and Q2 values of 0.95 and 0.79, respectively. These PLS-DA models were validated by the permutation method through 100 applications in which the Q2 values of permuted Y vectors were lower than the original ones and the regression of Q2 lines intersect at below zero. The score plot in **Fig. 9** shows a separation between JA-treated and control samples observed at 24 – 72 hours after elicitation. There is one outlier observed for the JA-treated samples after 48 hours elicitation, which distantly separates from its biological replicates. This could be due to either technical or biological variation of the sample, in which the latter is the most influential in many cases.

To get a better separation of the validated PLS-DA model, another supervised algorithm, orthogonal projection to latent structures-discriminant analysis (OPLS-DA) was applied to the same data. OPLS-DA is used as improvement of the PLS-DA method to separate two or more classes of multivariate data (Bylesjö et al. 2006; Westerhuis et al. 2010). In OPLS-DA, a regression model is calculated between the multivariate data and a response variable that only contains class information (Westerhuis et al. 2010). The advantage of OPLS-DA compared to PLS-DA is that a single component is used as a predictor for the class, while the other components describe the variation orthogonal to the first predictive component (Westerhuis et al. 2010). As shown in **Fig. 10,** OPLS-DA clearly distinguished between JA-treated and control samples after 24 – 72 hours of elicitation. The loading plot (**Fig. 11**) reveals that the JA-elicited cells were higher in aromatic and amino/organic acid regions, while the control cells were distinguished by sugars. Some of the compounds identified for the separation of JA-elicited cells were strictosidine, succinic acid, fumaric acid, malic acid, and leucine. Sucrose, glucose, and alanine were found higher in the control samples. The increased level of strictosidine and decreased level of sugars in JA-elicited cells suggest that sugars are used for the production of ATP and as carbon sources of plant defense compounds.

Instead of using PLS-DA, PLS can also be used to reduce the dimensionality of multivariate data and to find relations between X and Y matrices. In PLS-DA, a dummy Y-variables is created by assigned classes, while in PLS, additional data are included as Y-data sets; in this case time and elicitation. As for the PLS-DA, all samples from 0 – 72 hour time-points did not deliver a validated PLS model, whereas only the 24 – 72 hours samples deliver a good PLS model (**Fig. 12)**. Since the PLS has more than one Y-variable, bidirectional orthogonal PLS (O2PLS) was used in order to discriminate the cells by time and treatment.

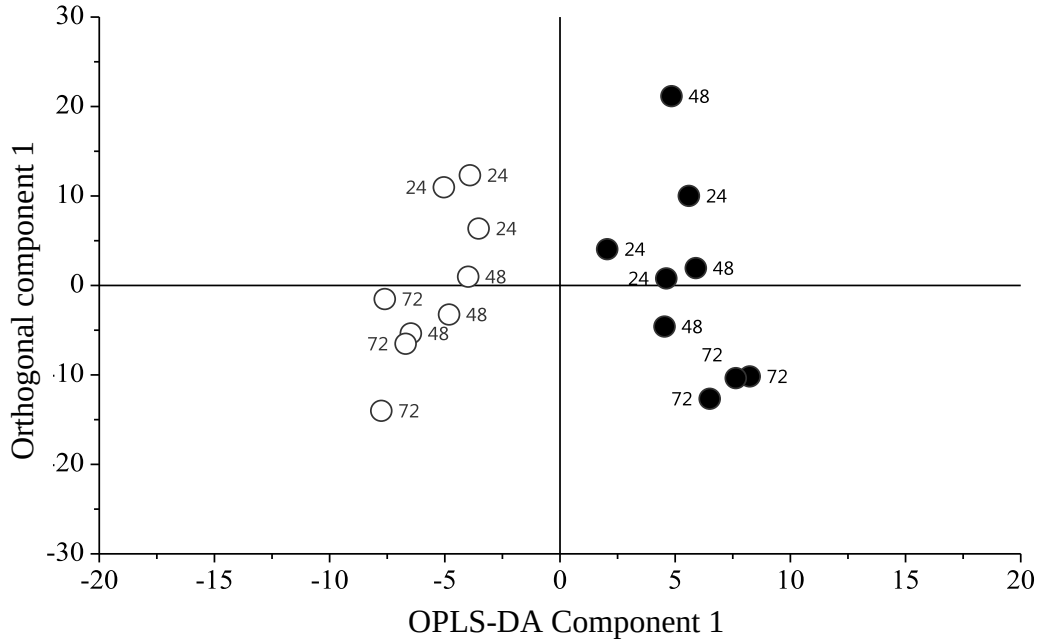


Fig. 10 Score plot of orthogonal projection to latent structures-discriminant analysis (OPLS-DA) of jasmonic acid-elicited (●) and control (○) samples of the CRPP cell line measured by ^{1}H-NMR. The numbers in the score plot are harvesting time (hour) after treatments.

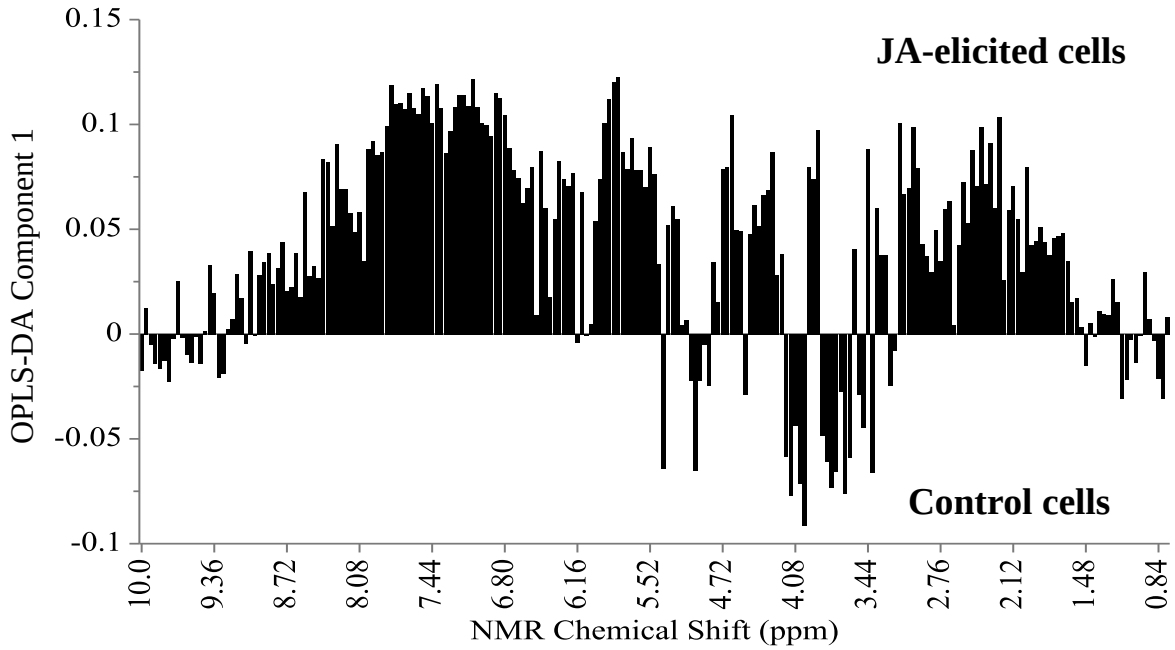


Fig. 11 Loading plot of orthogonal projection to latent structures-discriminant analysis (OPLS-DA) of jasmonic acid (JA)-elicited CRPP cells and control cells.

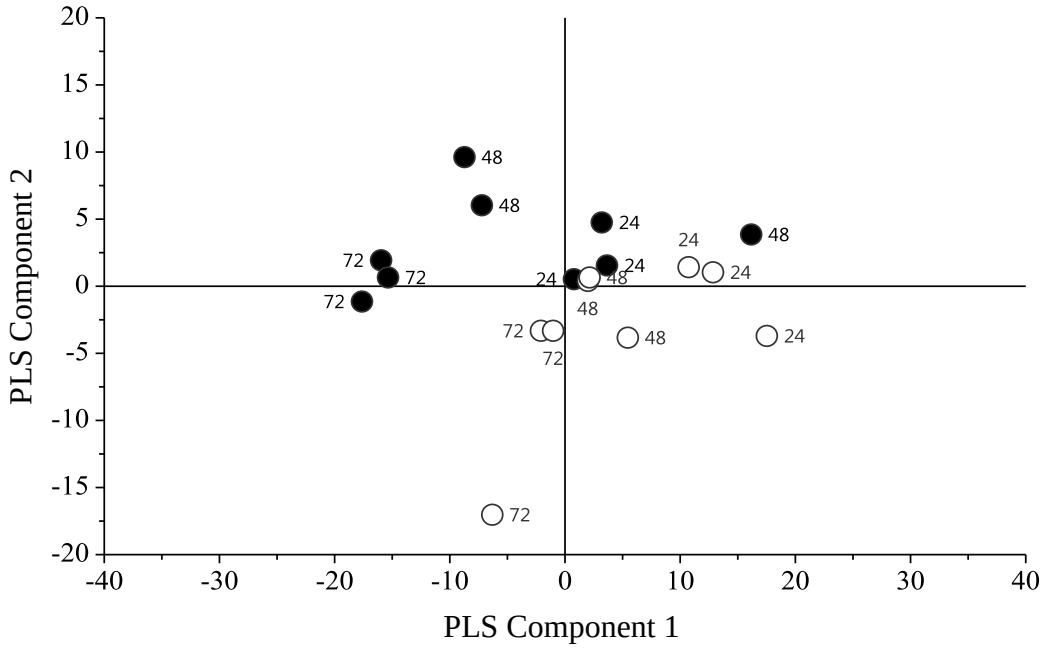


Fig. 12 Score plot of partial least squares (PLS) of jasmonic acid-elicited (●) and control (○) samples of the CRPP cell line measured by ^{1}H-NMR. The numbers in the score plot are harvesting time (hour) after treatments.

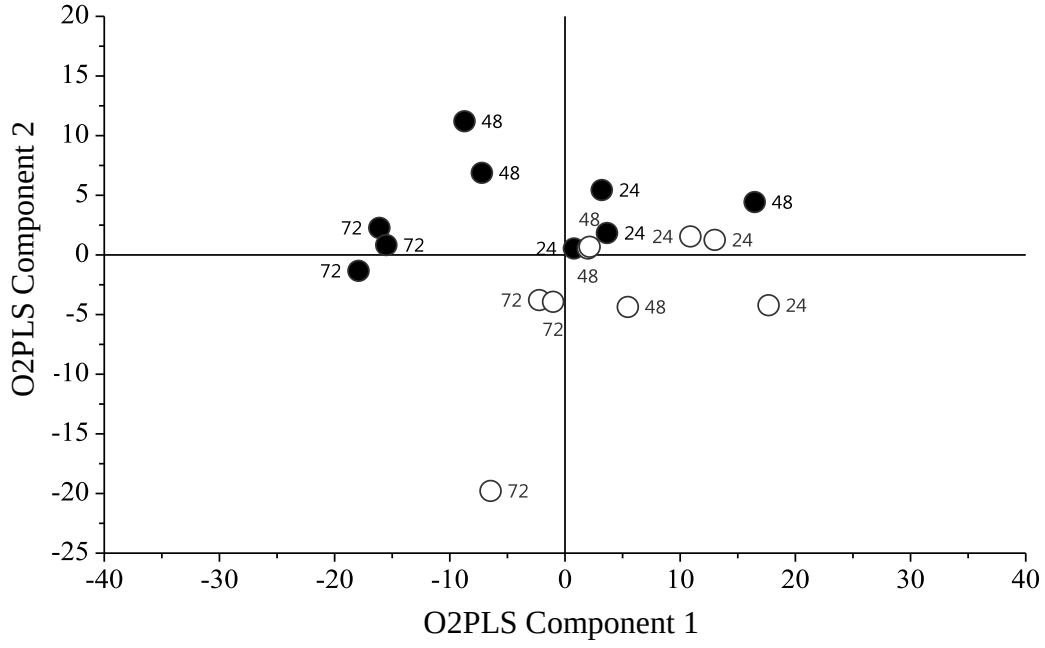


Fig. 13 Score plot of bidirectional orthogonal projection to latent structures (O2PLS) of jasmonic acid-elicited (●) and control (○) samples of the CRPP cell line measured by ^{1}H-NMR. The numbers in the score plot are harvesting time (hour) after treatments.

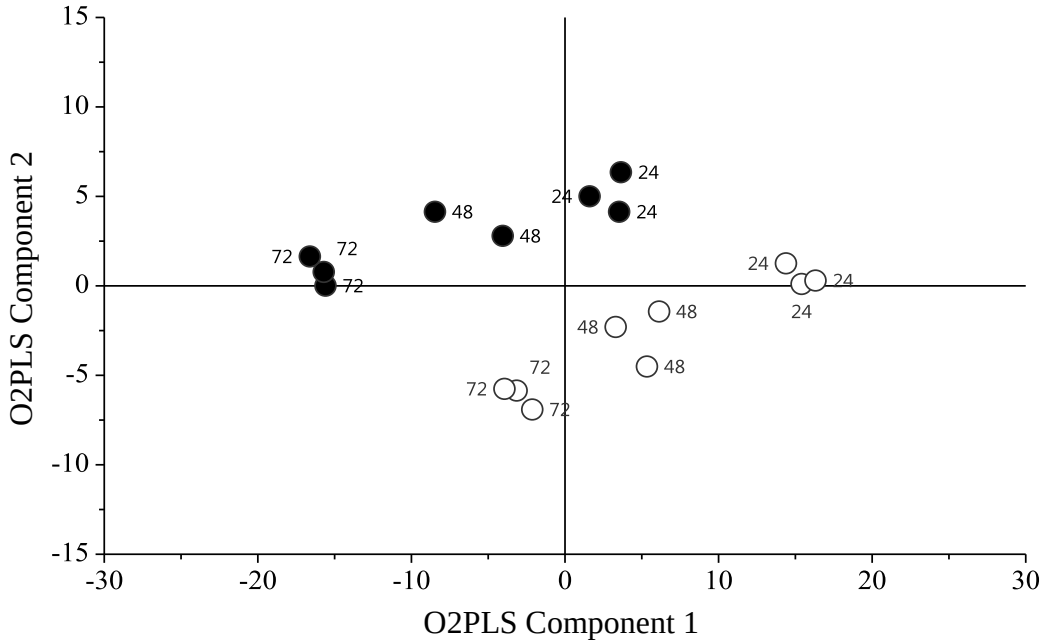


Fig. 14 Score plot of bidirectional orthogonal projection to latent structures (O2PLS) shows better separation of jasmonic acid-elicited (●) and control (○) samples after removing an outlier of JA-elicited sample at 48 hours. The numbers in the score plot are harvesting time (hour) after treatments.

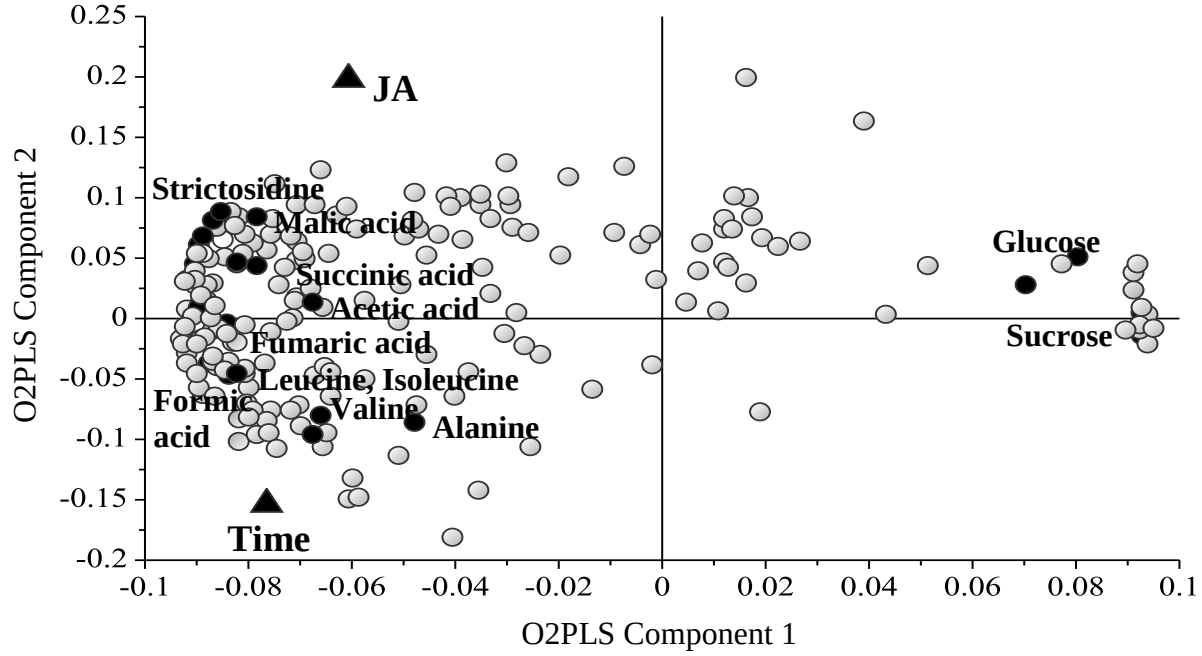
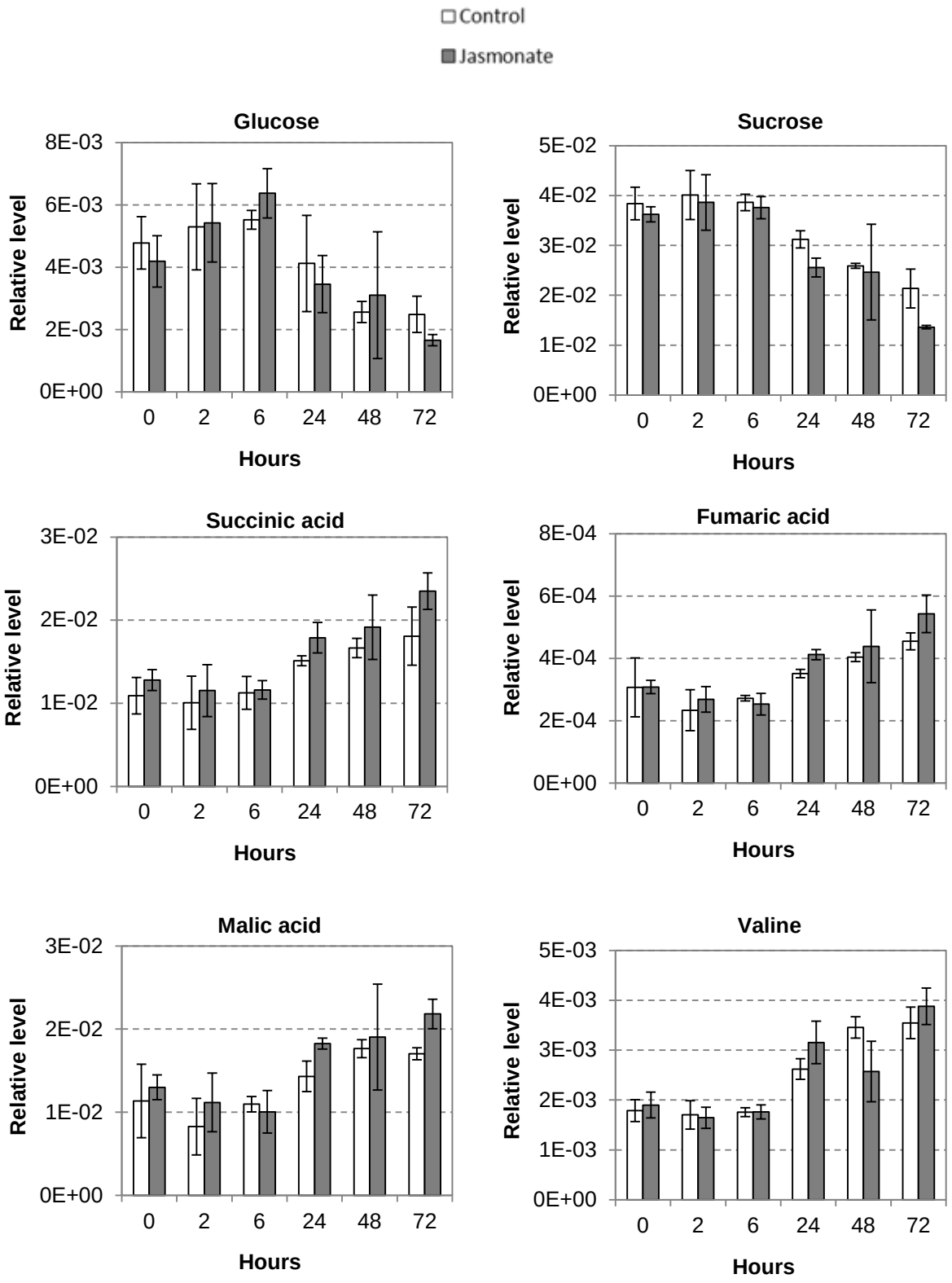


Fig. 15 Loading plot of bidirectional orthogonal projection to latent structures (O2PLS) shows relation of variables based on jasmonic acid (JA) treatment and cell age (time).

Nevertheless, the sample separation in O2PLS showed a similar pattern as in the PLS model **(Fig. 13)**. By removing the forementioned outlier of the 48-hours JA-elicited samples, the O2PLS model was improved **(Fig. 14).** The O2PLS loading plot (**Fig. 15**) shows that most of the metabolites were relatively increased through time except sugars. Strictosidine, malic acid, and succinic acid were most influenced by JA elicitation over the time period.

Relative levels of metabolites detected by NMR

Although multivariate data analysis, either supervised or unsupervised, is useful to reduce the dimensionality of a multivariate dataset and to identify differences or similarities among the samples, the time-course levels of individual metabolites involved in the samples discrimination are not presented. **Figure 16** shows the transient levels of several compounds identified from their characteristic signal in NMR data. As revealed in OPLS-DA and O2PLS results, sugars levels decreased by time and sucrose levels were significantly lower ($P < 0.05$) at 24 and 72 hours in the JA-elicited cells compared to the control. In contrast, Flores-Sanchez et al. (2009) found no significant difference of sucrose and glucose levels in methyl jasmonate-elicited *Cannabis sativa* cell suspension cultures if compared to their respective controls. Some components of the TCA cycle; succinic acid, fumaric acid, and malic acid were increased at 24 and 72 hours after elicitation, although only malic acid levels were statistically significant compared to the control ($P < 0.05$). A similar result was found in the cell suspension cultures of *C. sativa* in which fumaric acid level was induced after MeJA elicitation (Flores-Sanchez et al. 2009). The current results suggest that upon jasmonate elicitation, sugars were catabolized and TCA cycle was activated to make building blocks and generate chemical energy for various biosynthetic routes that are part of the defense response. The levels of valine remained statistically unaltered between JA-elicited and control cells at all time-points ($P < 0.05$). Leucine was found higher at 24 and 72 hour after elicitation in which the level at the former time-point was significantly increased ($P < 0.05$). Interestingly, the amino acid alanine was significantly decreased after 72 hour of elicitation ($P < 0.05$), which might be linked to the decreased levels of sugars. Liang et al. (2006) also found that glucose, sucrose, and alanine were decreased in MeJA treated *Brassica rapa* leaves compared to the control. In agreement to HPLC results, strictosidine levels were significantly increased at 24 and 72 hours after elicitation ($P < 0.05$). The level of loganic acid in JA-elicited cells increased at 24 hours ($P < 0.05$), whereas loganic acid levels were not significantly different between elicited and control cells at 72 hours after elicitation.



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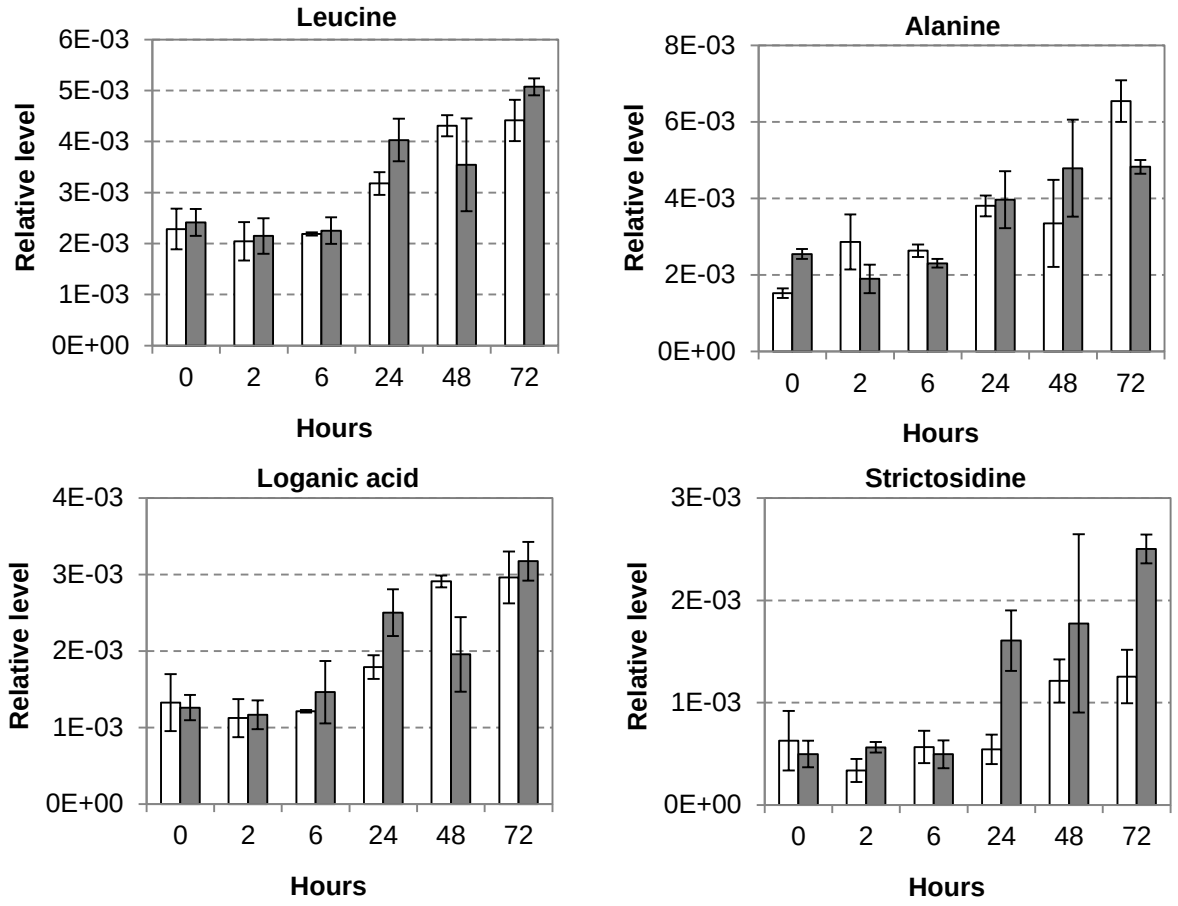

Fig. 16 Relative level of metabolites based on the mean area (^{1}H-NMR bucket data) of the resonance peak related to the metabolites. Results are average of three replicates ± standard deviation.

Conclusion

The effects of JA elicitation on primary and secondary metabolism in *C. roseus* cell suspension cultures were studied. **Figure 17** summarizes the metabolic effect of JA elicitation in this study. Elicited cells showed higher levels of TIA and carotenoids, while sterol accumulation was not affected, indicating that the flux of C5 units was only increased in the MEP pathway upon JA elicitation. For both carotenoid and TIA pathway, the available enzymatic machinery seems to have a large overcapacity, able to deal with in increased carbon flux from the MEP pathway. To increase TIA production, channeling the increased availability of the C5 units away from carotenoids to TIA would be an interesting strategy. This shows that a systemic study of the metabolic changes in cells can lead to novel approaches of metabolic engineering.

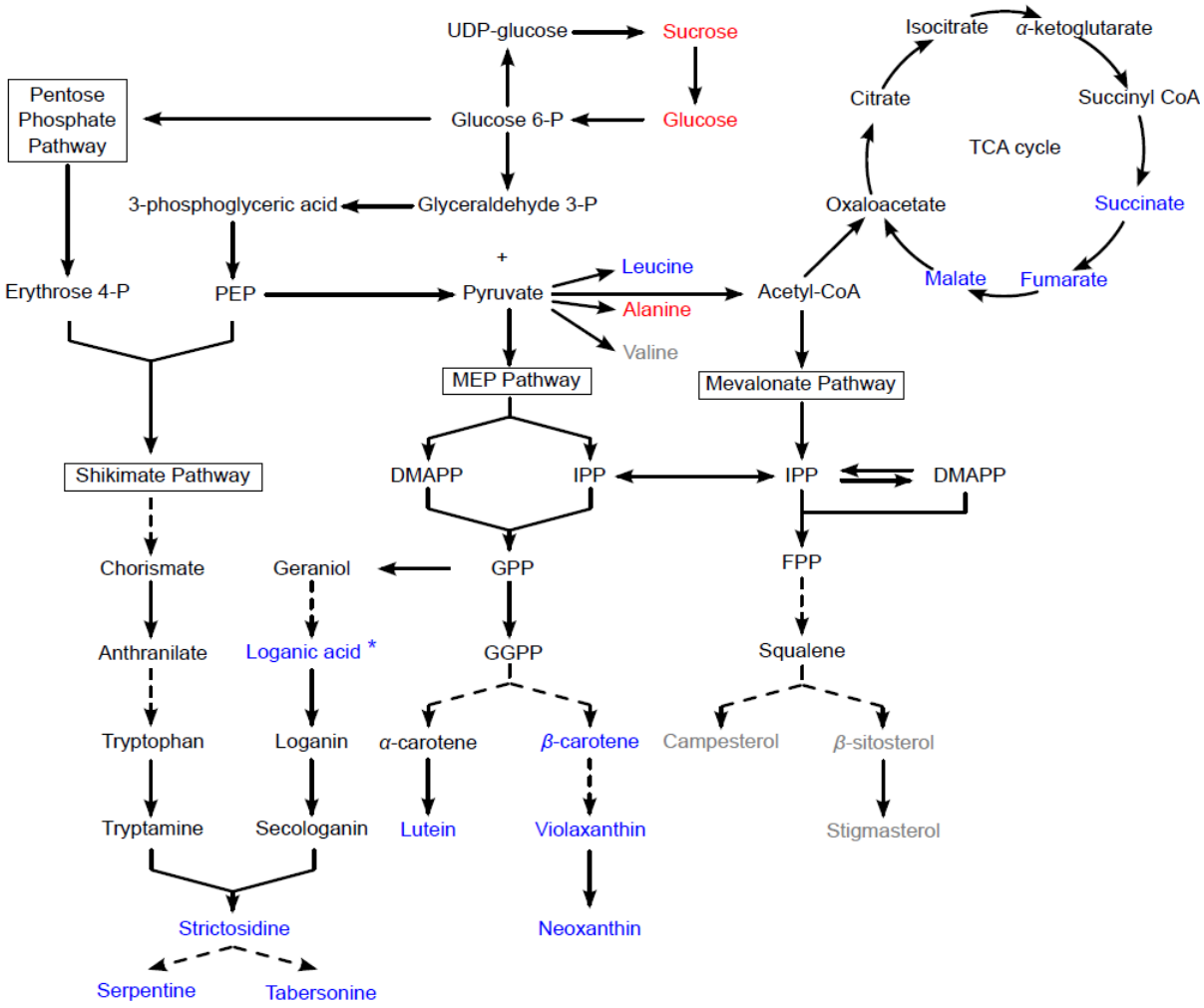

Fig. 17 Schematic summary of the results obtained in this study. The levels of the identified metabolites which were increased (blue), decreased (red), or relatively unchanged (grey) after jasmonic acid elicitation in *C. roseus* cell suspension cultures. *Loganic acid level increased at 24 hours after elicitation, whereas at 72 hours the level is about similar to the level of the control.

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