Retrobiosynthetic study of salicylic acid in Catharanthus roseus cell suspension cultures
Mustafa, N.R.

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Chapter 7

Metabolic profiling of *Catharanthus roseus* cell suspension cultures elicited with salicylic acid

N. R. Mustafa, H. K. Kim, Y. H. Choi and R. Verpoorte

Division of Pharmacognosy, Section Metabolomics, Institute of Biology

Leiden University, Leiden, The Netherlands

Abstract

Salicylic acid is a signal compound in systemic acquired resistance in plants. The effect of salicylic acid on the metabolic profile of *Catharanthus roseus* suspension cells in a time course (0, 6, 12, 24, 48 and 72 h after treatment) was studied using ^{1}H-NMR spectrometry and principal component analysis (PCA). The results of PCA showed that 25 µmol of sodium salicylic acid spiked into 100 mL of 5 days-old cell suspension cultures altered the metabolome compared with the non-treated (control) cells. The sugars (glucose and sucrose) were found in higher levels in the SA-treated cells than in the control cells. The sugars signals disappeared after 48 h both in the control- and treated cells. The dynamic levels of some aliphatic amino acids (alanine, isoleucine, leucine, threonine, valine) and some organic acids (aspartic acid and fumaric acid) were observed in the SA-treated cells. In the SA-treated cells, SA was detected at 0-24 h. It had disappeared after 48 h, but at the same time 2,5-dihydroxy-5-*O*-glucoside (already detected at 24 h in a relatively low level) reached the highest level. Other compounds detected both in the control- and treated cells were tryptamine, phenylalanine and a phenylpropanoid.

Keywords: *Catharanthus roseus*, cell suspension cultures, salicylic acid, elicitation, metabolic profiles, ^{1}H-NMR spectrometry and principal component analysis

7.1 Introduction

Catharanthus roseus is a terpenoid indole-alkaloid (TIA) producing plant. There are more than 100 TIAs found in this species, including the anti-tumor agents vincristine and vinblastine (dimeric) and the anti-hypertension agent ajmalicine. However, dimeric TIAs are produced by this species in relatively low amounts. In the past decades several efforts have been made to achieve a plant biotechnological production of these compounds. However, so far no economically feasible process could be developed (Verpoorte *et al*., 2002).

Secondary metabolites are involved in the interaction of a plant with its environment (e.g. biotic or abiotic stress). Upon a pathogenic attack, a plant is able to induce a defense system known as systemic acquired resistance (SAR), which involves salicylic acid (SA) as a signal compound (Ryals *et al*., 1996). However, a cross-talk with other signal compounds in SAR, e.g. jasmonate (JA) and/or ethylene, is also possible (Glazebrook *et al*., 2003). Identification of the SA-dependent pathways and SA-independent pathways is important to gain more insight in SAR and the production of secondary metabolites by plants.

Activation of SA-dependent SAR genes results in the production of acidic PR proteins and other metabolites, providing a broad spectrum of antimicrobial-activities (Ryals *et al*., 1996). The level of SA increases when a plant is attacked by pathogens e.g. tobacco mosaic virus (Verberne *et al*., 2000) or *Pythium aphanidermatum* (Chapter 3 of this thesis). In addition to SA, the level of 2,3-dihydroxybenzoic acid (2,3-DHBA) (Moreno *et al*., 1994a; Budi Muljono *et al*., 2002) and tryptamine (Moreno *et al*., 1996) increased in *C. roseus* suspension cells upon elicitation with *Pythium* extract. However, *Pythium* extract is a mixture of compounds, which may activate different parts of the SAR pathways employing different signaling compounds. Therefore, the metabolites affected by salicylic acid alone needs to be investigated.

^{1}H-NMR spectrometry in combination with PCA has shown to be a powerful method for studying metabolism in plants including *C. roseus* (Kim *et al*., 2006). This method was applied to *C. roseus* for discriminating metabolites between healthy leaves and phytoplasma-infected leaves (Choi *et al*., 2004), and also within *Tabernaemontana divaricata* cell suspension cultures before- and after cryopreservation (Suhartono *et al*., 2005).

In this study, we analyzed the effect of SA on the metabolites profiles of *C. roseus* cell suspension culture using ^{1}H-NMR and PCA.

7.2 Materials and methods

7.2.1 Chemicals

7.2.1.1 Chemicals used in suspension culture

The chemicals used in Macro Murashige & Skoog (M&S) salts: $CaCl_2$ (min. 99%), KH_2PO_4 (min. 99.5%), KNO_3 (min. 99%) and NH_4NO_3 (min. 99%) were purchased from Merck (Darmstadt, Germany) and $MgSO_4$ was obtained from OPG Farma BUVA B.V.(Uitgeest, The Netherlands). The chemicals used in Micro M&S salts: H_3BO_3, $MnSO_4.H_2O$, Na_2EDTA, $ZnSO_4.7H_2O$ (Merck) and $FeSO_4.7H_2O$ (Brocades-ACF groothandel NV, Maarssen, The Netherlands) were dissolved in one solution, whereas $CoCl_2.6H_2O$, $CuSO_4.5H_2O$, KI and $NaMoO_4.2H_2O$ (Merck) were dissolved separately due to problems of solubility. Glycine (99.7%) and nicotinic acid (99.5%) were from Merck. D(+)-Glucose (> 99.0%) was from Fluka Chemie (Buchs, Germany). myo-Inositol (> 99.0%) was from Duchefa Biochemie (Haarlem, The Netherlands). Pyridoxine-HCl was from Sigma-Aldrich Chemie (Steinheim, Germany) and thiamine-*di*-HCl was from Janssen Chimica (Geel, Belgium).

7.2.1.2 Chemicals used in extraction and NMR analysis

CH_3OH-d_4 was obtained from C.E. Saclay (Gif-Sur-Yvette, France) and KH_2PO_4 was from Merck. D_2O (99.00%) was purchased from Cambridge Isotope Laboratories Inc. (Miami, FL, USA).

7.2.2 Plant cell cultures

Catharanthus roseus line A12A2 was grown in Murashige and Skoog (M&S) liquid medium (Murashige and Skoog, 1962) without growth hormone and was supplemented with 2% of D(+)-monohydrate glucose as a carbon source. The cells were grown in 250 mL-Erlenmeyer flasks containing 100 mL medium, cultivated at 24-25 °C under continuous light (500-1500 lux) on a shaker at 100 rpm, and subcultured every week by adding the same amount of fresh medium into the cell

cultures for maintenance. For the experiment, the suspension cells were subcultured into 100 mL-Erlenmeyer flasks (each containing 50 mL medium) in the same cultivation conditions for 5 days before elicitation.

7.2.3 Elicitation and harvesting cells

A solution of 0.5 M sodium-salicylic acid was filtered using a 0.2 μm-membrane filter before adding (50 μl) into a 100 ml-flask containing 50 mL cell suspension culture to provide the concentration of 0.5 mM of sodium-SA in the culture. As control, 50 μl sterilized-water was added into 50 mL cell suspension culture. The elicited cells as well as the control cells were harvested at time zero, 6 h, 12 h, 24 h, 48 h and 72 h after the addition of the sodium-salicylic acid or the sterilized-water. Three flasks were used for each time point (n = 3) both for the elicited cells and the control cells. In the harvesting step, the cells from a flask were rinsed twice with 100 mL de-ionized water, vacuum filtered using a P2 glass-filter, put in a 10 mL-plastic tube, weighed and stored in -80°C. The frozen-cells were freeze-dried in 48 h.

7.2.4 Extraction

Fifty mg of freeze-dried cells of each flask were put in a 2 mL-micro tube. Seven hundred fifty μl of CH_3OH-*d₄* and 750 μl of KH_2PO_4-d_2 buffer in D_2O (pH 6.0) were used as the extraction solvent. The mixture was vortexed (2,500 rpm, 1 min) using a Vibrofix VF1 electronic vortex (IKA, Staufen, Germany), followed by sonication (20 min) in an ultrasonic bath and centrifugation (13,000 rpm, 15 min) using a BHG HermLe Z 231 M centrifuge (B. HermLe, Gosheim, Germany). The supernatant was transferred into a 5 mm-NMR tube for NMR measurement.

7.2.5 NMR measurements

^{1}H-NMR and J-resolved spectra were recorded at 25 ºC on a 400 MHz Bruker AV-400 spectrometer operating at proton NMR frequency of 400.13 MHz. CH_3OH-d_4 was used as an internal lock. Each spectrum consisted of 128 scans requiring 10 min acquisition time with the following parameters: 0.25 Hz/point, pulse width (PW) = 45º (6.6 μsec), and relaxation delay (Dl) = 2.0 sec. A presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz

and the spectra were zerofilled to 32 K points. The window functions were optimized for the analysis. The resulting spectra were manually phased and baseline corrected, and calibrated to TSP at 0.0 ppm, always using XWIN NMR (version 3.5, Bruker). Two dimensional J-resolved ^{1}H-NMR spectra were acquired using 8 scans per 32 increments that were collected into 16 K data points, using spectral widths of 5.208 KHz in F2 (chemical shift axis) and 50 Hz in F1 (spin-spin coupling constant axis). A 1.0 sec relaxation delay was employed, giving a total acquisition time of 14.52 min. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions prior to double complex FT. J-resolved spectra tilted by 45º were symmetrized about F1 and then calibrated, always using XWIN NMR (version 3.5 Bruker). Data were exported as the 1 D projection (F2 axis) of the 2D J-resolved spectra.

7.2.6 Data analysis

The ^{1}H-NMR and F_2-projected J-resolved spectra were automatically reduced to ASCII files using AMIX (v. 3.7, Bruker Biospin). Spectral intensities were scaled to TSP and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ -0.40 - δ 10.00. Principal component analyses (PCA) were performed with the SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden).

7.3 Results and discussion

PCA was applied to the ^{1}H-NMR spectra of control- and SA-treated cells harvested at 0 h, 6 h, 12 h, 24 h, 48 h and 72 h after treatment. The score-plot is shown in Figure 7.1. The combination of PC1 and PC2 was able to differentiate all the groups evaluated (the control- and treated cells at different time points). The elicited-groups is clearly separated from the control-groups mainly by PC2, at which the elicited groups give a positive effect and the control groups provide a negative effect to PC2. Most of the control groups of interval 0-24 h is not differentiated by PC1 except the 6 h-control cells which shows a positive effect, whereas the rest of the control groups (48-72 h) provide a negative effect to PC1. The negative effect observed for 48 h and 72 h of control groups represent changes in growth stage of the cells (age).

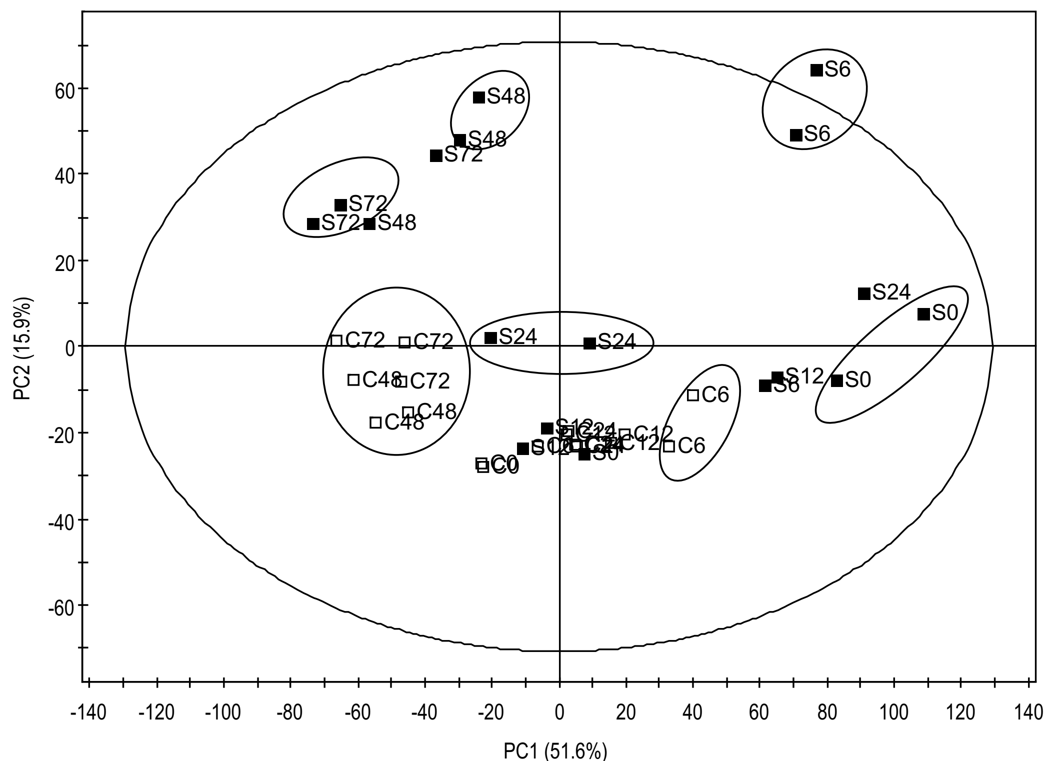

Figure 7.1. The score plot of PCA of ^{1}H-NMR data of *Catharanthus roseus* cells extracted with CH_3OH-d_4-KH_2PO_4 in H_2O-d_2 pH 6.0 (1 : 1). C: control cells, S: SA-treated cells, the number after the symbol shows the observation time (hour) after treatment.

Among the elicited groups, the 0 h treated-cells gave the highest positive effect to PC1 and the positive effect decreases through time. This indicates that there is an immediate change in e.g. pH, oxidative-burst and glucosidases activity after elicitation if compared to the control groups (0 h, 12 h and 24 h), after which it normalizes again (24 h after elicitation) to give a second change on the longer term (48-72 h after elicitation), which is probably dependent on gene-induction. Either SA or JA or other signal compounds can induce such change. The change in metabolic profile in this study has a comparable pattern with the change in gene-expression or enzyme activity in *C. roseus* suspension cultures treated by JA or *Pythium* extract observed by other groups (van der Fits and Memelink, 2001; Pasquali *et al*., 1992 and Moreno *et al*., 1996). Van der Fits and Memelink (2001) showed that elicitation of *C. roseus* cells with methyl-jasmonate (MeJA) rapidly induced a regulator gene *ORCA3* already within 0-2 h with the highest level at 0.5 h, whilst the mRNA of the structural

genes e.g. *TDC* and *STR* responsible for tryptophan decarboxylase (TDC) and strictosidine synthase (STR) were strongly induced later, 2-8 h after elicitation with the maxima at 8 h. Also, Pasquali *et al*. (1992) found that *C. roseus TDC* and *STR* were induced 8 h after elicitation with yeast- or *Pythium aphanidermatum* extract. Moreno *et al*. (1996) showed that increased-activity of anthranilate synthase (AS) from 2 to around 8 nkat/L occurred more than 10 h after elicitation, whereas TDC activity increases from around 20 to 90 nkat/L between 20-30 h after elicitation with *Pythium* extract. Strictosidine synthase and strictosidine-β-glucosidase activities were slightly increased compared to control cells. Budi Muljono (2001) observed a metabolic change through time in *C. roseus* cells elicited by *Pythium* extract. They found that the highest increase of the level of endogenous SA in the cells occurred 8 h after elicitation, whilst the highest increase of the level of 2,3-DHBA in the cells was found 20 h after elicitation. The highest level of 2,3-DHBA in the medium was observed at 24 h after elicitation. Chapter 6 of this thesis discusses the possibility of a different time and/or localization of the biosyntheses of both compounds shown by their different labeling patterns. All these previous results present evidence for the occurrence of different phases of cell metabolism upon elicitation. The phases can be distinguished as biochemical changes (phase 1), altering gene expression (phase 2) and switching on the defense response/metabolites (phase 3). The present study shows indeed such a pattern of different phases in the *C. roseus* cells metabolism upon elicitation.

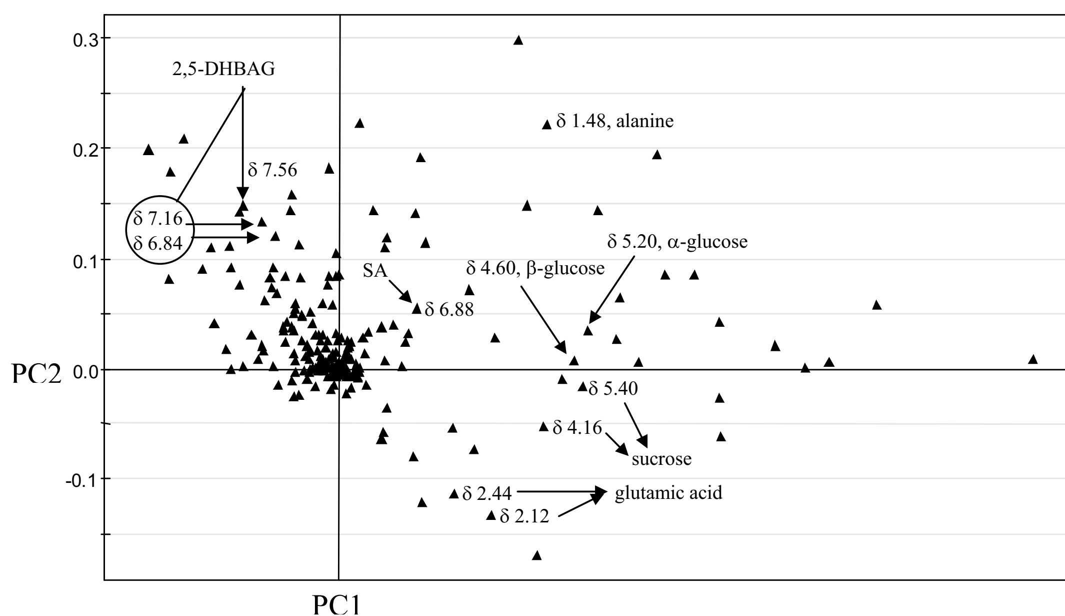

Figure 7.2. Loading plot of PC1 and PC2 of ^{1}H-NMR data of control- and SA-treated cells of *Catharanthus roseus*.

To further investigate the metabolic changes, the discriminating peaks were identified from the loading plot of PC1. The signals of some compounds having either positive or negative effect in the loading plots of PC1 vs PC2 are shown in Figure 7.2. Table 7.1 shows the compounds detected in the control- and/or SA-treated cells.

Table 7.1. The chemical shifts (δ in ppm) and coupling constants (*J*) of some compounds detected on the ^{1}H-NMR and J-resolved ^{1}H-NMR spectra (CH_3OH-d_4-KH_2PO_4 in H_2O-d_2 pH 6.0, 1:1) of control- and SA-treated cells of *Catharanthus roseus* suspension culture. *s* = singlet, *d* = doublet, *t* = triplet, *m* = multiplet.

Compound	**Chemical shift (ppm) and coupling constant (Hz)**
Leucine	δ 0.97 (*d*, *J* = 6.5 Hz); δ 0.98 (*d*, *J* = 6.7 Hz)
Isoleucine	δ 0.95 (*t*, *J* = 7.5 Hz); δ 1.02 (*d*, *J* = 6.8 Hz)
Valine	δ 1.00 (H-γ, *d*, *J* = 7.0 Hz); δ 1.05 (H-γ', *d*, *J* = 7.0 Hz)
Threonine	δ 1.33 (H-γ, *d*, *J* = 6.6 Hz)
Alanine	δ 1.48 (H-β, *d*, *J* = 7.2 Hz)
Glutamic acid	δ 2.46 (H-γ, *m*), δ 2.13 (H-β, *m*)
Aspartic acid	δ 2.82,(H- , *dd*, J_1 = 8.1 Hz, J_2 = 17.0 Hz); δ 2.95 (H- , *dd*, J_1 = 3.8 Hz, J_2 = 16.8 Hz); δ 3.94 (H- , *dd*, J_1 = 3.8 Hz, J_2 = 8.4 Hz)
β-Glucose	δ 4.58 (H-1, *d*, *J* = 7.8 Hz)
α-Glucose	δ 5.18 (H-1, *d*, *J* = 3.8 Hz)
Sucrose	δ 5.40 (H-1, *d*, *J* = 3.8 Hz); δ 4.17 (H-1', *d*, *J* = 8.6 Hz)
A phenylpropanoid	δ 6.51 (H-7, *d*, *J* = 16.0 Hz); δ 7.02 (H-2 & H-6, *s*)
Fumaric acid	δ 6.54 (*s*)
Tryptamine	δ 7.13 (*t*, *J* = 7.4 Hz); δ 7.20 (*t*, *J* = 7.5 Hz), δ 7.29 (*s*); δ 7.47 (*d*, *J* = 8.1 Hz); δ 7.64 (*d*, *J* = 7.9 Hz)
Phenylalanine	δ 7.36 (*m*)
Salicylic acid (2- Hydroxybenzoic acid)	δ 6.88 (H-3, *d*, *J* = 8 Hz); δ 6.90 (H-5, *t*, *J* = 7.5 Hz); δ 7.37 (H-4, *t*, *J* = 7.5 Hz); δ 7.81 (H-6, *d*, *J* = 8 Hz)
Gentisic acid glucoside (2,5-Dihydroxybenzoic acid glucoside)	δ 6.83 (H-3, *d*, *J* = 9 Hz); δ 7.17 (H-4, *dd*, J_1= 9 Hz, J_2= 3 Hz); δ 7.56 (H-6, *d*, *J* = 3 Hz); δ 4.88 (H-1', *d*, *J* = 7.5 Hz)

J-resolved ^{1}H-NMR spectra showing the splitting pattern of peaks based on the coupling-constants (J), were also used to confirm the identification of the metabolites in Table 7.1 (e.g. Figure 7.3 and 7.4).

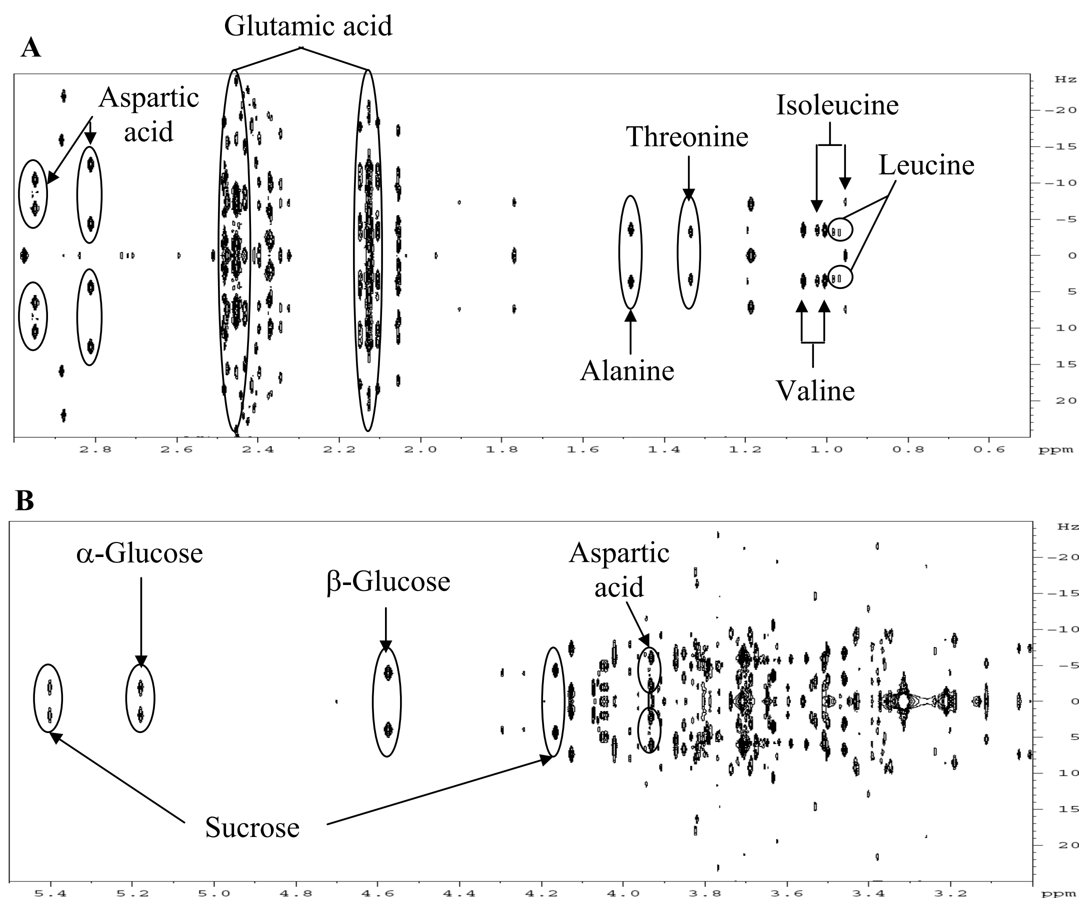

Figure 7.3. Some metabolites signals in the aliphatic region (A) and sugars region (B) of J-resolved ^{1}H-NMR spectrum (CH_3OH-d_4-KH_2PO_4 in H_2O-d_2 pH 6.0, 1 : 1) of the extract of *Catharanthus roseus* cells 12 h after elicitation with sodium-SA.

Subsequently, the signals of some metabolites in the ^{1}H-NMR spectra of the samples were quantified using a ratio to the intensity of the internal standard (TSP) signal in order to observe the changes in metabolite levels in the time course (Figure 7.5 and 7.6).

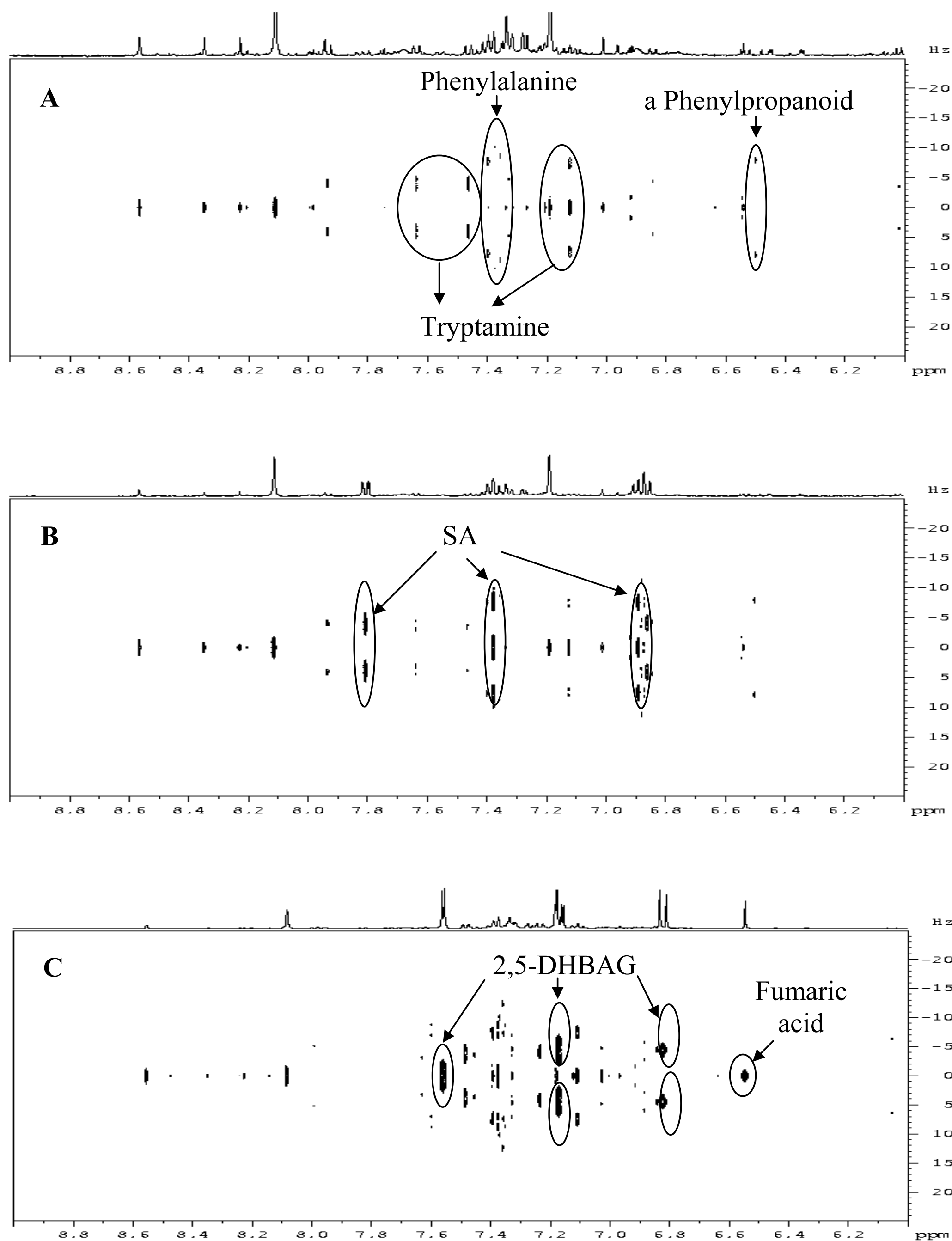

Figure 7.4. The signals of some compounds in the aromatic region of J-resolved ^{1}H-NMR spectra of *Catharanthus roseus* cells extracted with CH_3OH-d_4-KH_2PO_4 in H_2O-d_2 pH 6.0 (1 : 1). A: 6 h-control cells, B: 6 h-elicited cells, C: 48 h-elicited cells. SA= salicylic acid, 2,5-DHBAG= 2,5-dihydroxybenzoic acid glucoside.

The sugars (glucose and sucrose) provided a positive effect in PC1 (Figure 7.2), which consisted of the observation groups of 0 h – 24 h of both control- and treated cells. The changes through time due to growth are for both control and elicited cells represented mainly by PC1. In the SA-treated cells, the highest levels of the sugars were found at 0 h and these levels subsequently decreased to zero after 72 h. The control cells showed an increased-level of sugars at 6 h compared to 0 h and it further decreased also to zero after 72 h (Figure 7.5 A-B). It seems that in few minutes after the addition of sodium-SA (0 h), the soluble sugar pool in the cells increased probably for providing precursors and energy (ATP) for the cells response to elicitation. Increased levels of sugars were also reported to occur for example in *C. roseus* intact plants infected by phytoplasma (Choi *et al*., 2004) or potato leaves and tubers under different stress conditions (Świędrych *et al*., 2004). The latter group showed that increased levels of glucose, fructose and sucrose were a result of mobilization of starch initiated by catecholamines under stress conditions. The study was driven by the mobilization of glycogen by catecholamines (stress-hormones) as found in mammalian cells. In wild type and transgenic potato leaves under stress conditions, increased-levels of dopamine and norepinephrine paralleled the increased activities of tyrosine-hydroxylase (TH), tyrosine decarboxylase (TD) and L-dopa decarboxylase (DD) (enzymes at initial steps of catecholamine synthesis), and are accompanied by decreased-levels of normetanephrine (a catabolic product of norepinephrine). The authors also found a positive correlation between total SA and dopamine or norepinephrine in TD-overexpressing potato leaves. Dopamine and norepinephrine were detected by GC at levels of 0-9 μg/g and 0-30 μg/g FW plant materials. However, in *C. roseus* cells we could not find the catecholamines signals in the NMR spectra, which might be due to the lower sensitivity of NMR compared with GC-MS. Thus, increased levels of sugars can be considered as a common phenomenon occuring in cells undergoing stress, which could be aimed to provide precursors for both primary- and secondary metabolites, energy (e.g. ATP, GTP) and enzyme's co-factors (e.g. NADH) through some common pathways like glycolysis and the Krebs cycle. Besides, glucose is also necessary for detoxification of compounds by glycosylation (reviewed by Yamane *et al*., 2002). Depletion of sugars was found in 48-72 h control cells (Figure 7.5 A-B). Almost no sugar was left in the *C. roseus*

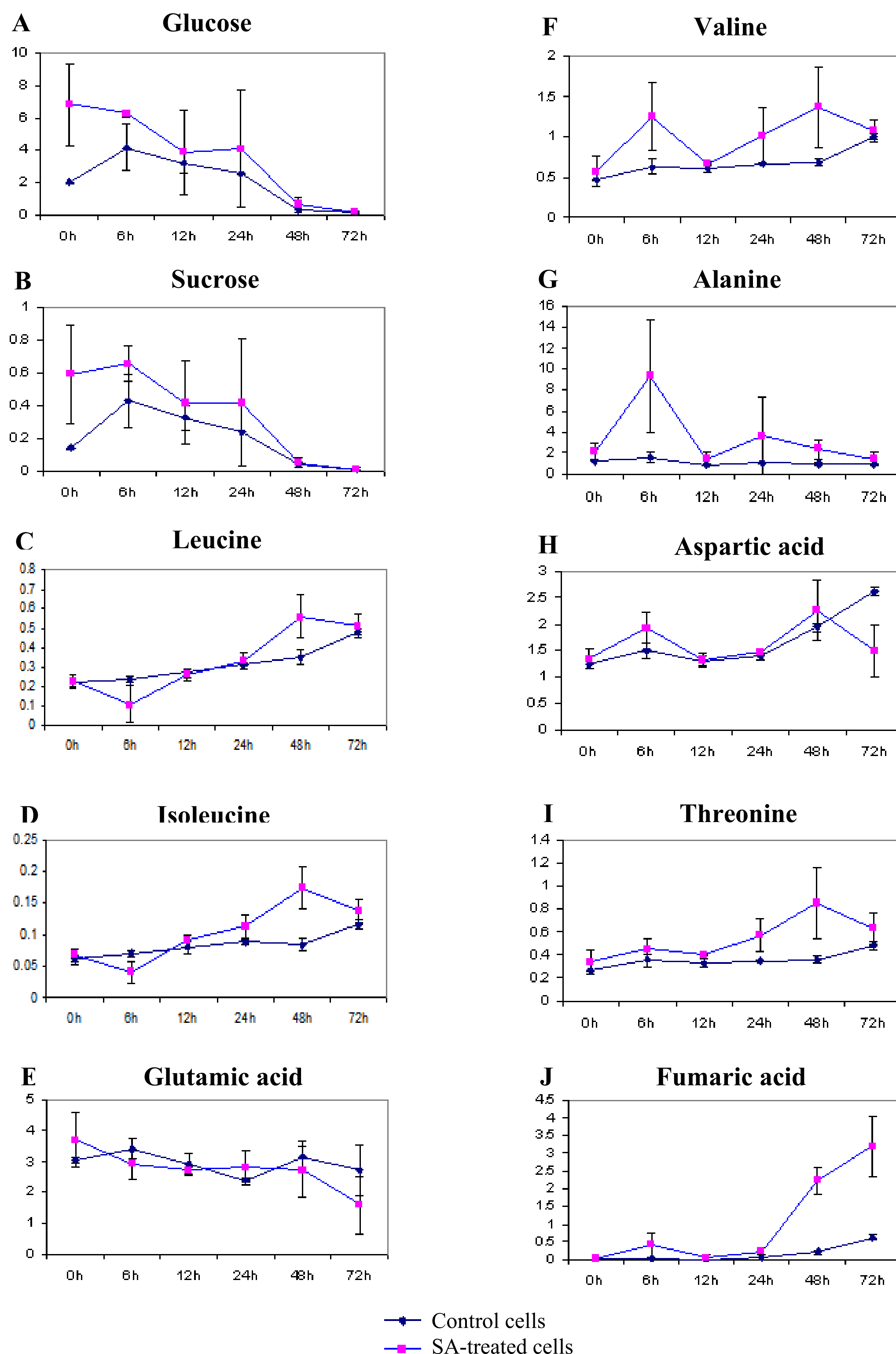

Figure 7.5. Time course (0-72 h) of the relative levels of sugars, some aliphatic amino acids and organic acids in the control- and SA-treated cells of *Catharanthus roseus* determined by a comparison of the peak-integrals of the compounds to the peak-integral of trimethylsilyl-propionate/ TSP (100%).

A12A2 cells 7 days after subculturing. This may explain the (more or less) stable fresh-weight of *C. roseus* cells observed after the 5th day of subculturing (Budi Muljono, 2001) when the cell culture growth stops.

Besides the levels of sugars, Figure 7.5 shows also the dynamic levels of some aliphatic amino acids and organic acids in the SA-treated cells observed in a time course of 0-72 h. In the SA-treated cells, maxima were shown at 6 h by valine, alanine, aspartic acid and fumaric acid, whilst at the same time minima were observed for leucine and isoleucine. At 0-6 h after the elicitation with SA, the pyruvate pool might increase through glycolysis, the Krebs cycle and the catabolism of leucine and isoleucine, which increases also the levels of valine, alanine, aspartic acid and fumaric acid. At 12 h after elicitation, the levels of the amino acids and organic acids (leucine, isoleucine, valine, threonine, aspartic acid and fumaric acid except glutamic acid and alanine) were back again to the initial levels, but subsequently increased again to reach another maximum at 48-72 h. In the range of 12-48 h, the pyruvate pool probably increased again for providing the precursors for the synthesis of secondary metabolites. The biosynthesis of some amino acids and organic acids is shown in Figure 7.7.

Glutamic acid was observed in the control cells almost at the same level during the whole time course, whereas in the time course of the elicited cells a slowly decreasing level of this compound was observed (Figure 7.5 E). Glutamate is necessary for ammonia (NH_3) assimilation to prevent the build-up of the toxic ammonia in the cell, which is produced by the reduction of nitrate (NO_3^-), nitrogen fixation or from the catabolism of other amino acids. Ammonia assimilation by glutamate leads to glutamine, which can be transaminated to obtain again glutamate when the amide amino group of glutamine is transferred to other compounds. The responsible enzymes (glutamine synthetase, EC 6.3.1.2 and glutamate synthase EC 1.4.1.13 or EC 1.4.7.1) form the glutamate synthase cycle. Glutamate can also be converted to proline and arginine. The energy and carbohydrates level in the cell/tissues are the most important factors for regulation of the N fluxes through the assimilation pathways (Miflin and Lea, 1982). Glutamine acts also as the amido donor to convert chorismate into anthranilate, a precursor of tryptophan (reviewed by Bongaerts, 1998).

Fumaric acid is present at low level at 0-24 h in the control cells, and the level slightly increases at 48-72 h. Both the control- and the elicited cells showed almost

the same (low) level of this compound in the range of 0-24 h (Figure 7.5 J). However, in the elicited cells the level increased significantly at 48 h and 72 h, probably as storage product due to a down-regulation of the Krebs cycle. Fumaric acid is an intermediate in the Krebs cycle, which can be converted into malic acid and subsequently to oxaloacetate. Phosphoenolpyruvate (PEP), a precursor of shikimate and chorismate, can also be formed from oxaloacetate. It is known that some enzymes in the Krebs cycle are down regulated if the level of energy (ATP) in the cell is already high, thus no excessive oxaloacetate would be produced for entering the Krebs cycle (Berg *et al*., 2002) and a high-level of fumaric acid might occur as a consequence.

Other compounds detected in the aromatic region were tryptamine, phenylalanine and a phenylpropanoid (Figure 7.4 A). The changes of the levels of these compounds in the time course are shown in Figure 7.6. Tryptamine was found at the highest level in the SA-treated cells at 0 h, but the compound had disappeared after 24 h. In the control cells, a similar pattern was found for the first 24 h but it was detected again at 48 and 72 h. Tryptamine, a precursor of terpenoid indole alkaloids, is a conversion product of tryptophan by the enzyme tryptophan decarboxylase.

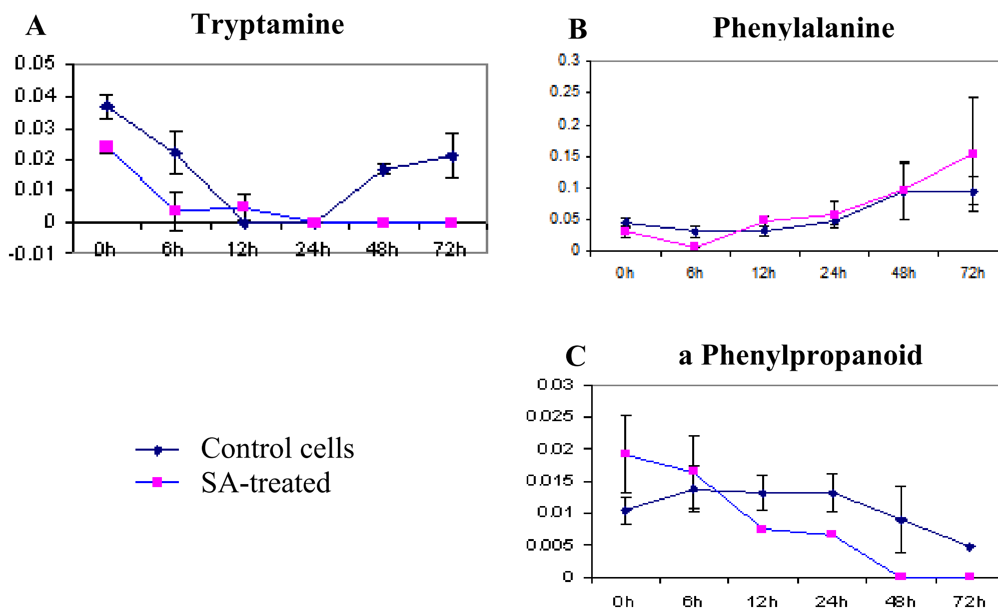

Figure 7.6. The levels of tryptamine (A), phenylalanine (B) and a phenylpropanoid (C) in the control- and SA-treated cells of *Catharanthus roseus* in a time course (0-72 h) determined by a comparison of the peak-integrals of the compounds to the peak-integral of TSP (100%).

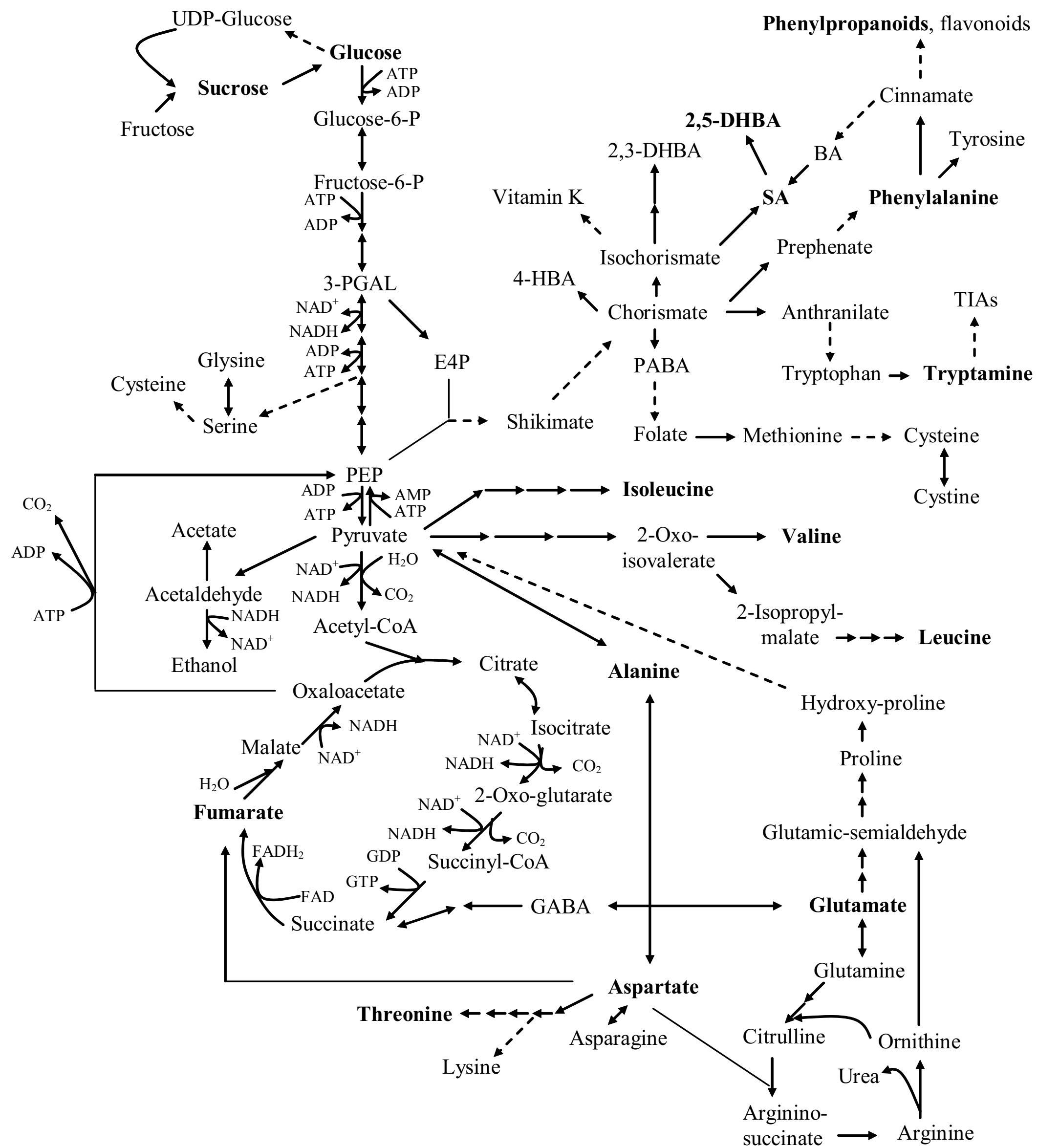

Figure 7.7. Glycolysis, The Krebs cycle and the biosynthetic pathways of some amino acids, organic acids, SA and 2,5-DHBA.

Phenylalanine was detected in both control and SA-treated cells. In the SA-treated cells the minimum level of this compound occurred at 6 h, subsequently the level increased again (16-fold) to reach the highest level at 72 h.

A signal detected at δ 6.51 (*d*, J = 16 Hz) was most probably the H-7 signal of a phenylpropanoid. This signal represented a relatively low-level of the compound observed in both control- and SA-treated cells. In the SA-treated cells, it disappeared after 48 h. Budi Muljono *et al*., 1998 reported the presence of cinnamic acid in *C. roseus* suspension cell cultures. However, based on a comparison with the NMR-spectra of the standard compounds we could not find the signals of the C6C3 cinnamic acid, coumaric acid, caffeic acid or ferulic acid in the NMR spectra of the control- and SA-treated cells. The signal at δ 6.51 (*d*, J = 16 Hz) and the presence of a singlet at δ 7.00 were almost similar with the signals of the *trans*-sinapic acid glucoside standard (δ 6.47, *d*, J = 16 Hz; δ 6.98, *s*; δ 7.32, *d*, J = 16 Hz). However, we could not confirm the signals as those of *trans*-sinapic acid glucoside because of the lack of a doublet with J = 16 Hz and also the HMBC spectrum did not support such a structure.

There was no SA detected in the NMR spectra of the control cells. In the SA-treated cells, relatively weak SA-signals were already detected at 0 h (data not shown). The SA-signals (Figure 7.4 B) appeared at 6-24 h but disappeared again after 48 h. In the NMR solvent (CH_3OH-d_4 : KH_2PO_4 in H_2O-d_2 pH 6.0, 1:1) the signals of H-5 (triplet) appeared at higher ppm than H-3 (doublet), which is the reversed of their positions in the spectra in CH_3OH-d_4 solvent (chapter 6 of this thesis). The presence of a relatively low level of SA already in 0-h SA-treated cells could be due to the rapid absorption of SA by the cells in a few minutes after treatment (± 3 min, including harvesting and washing cells with water). Though the harvested-cells were directly washed twice with 100 mL of water, the presence of a small amount of non-absorbed SA remaining on the cell walls might also be possible.

There were no 2,3-dihydroxybenzoic acid (2,3-DHBA) signals detected in the spectra of all observations, but we observed the presence of 2,5-dihydroxybenzoic acid glucoside (2,5-DHBAG) in the ^{1}H-NMR spectra of the SA-treated cells 48-72 h after treatment. This was confirmed by the ^{1}H-NMR spectrum of 2,5-DHBA standard (data not shown), the J-resolved ^{1}H-NMR (Figure 7.4 C), the NMR-spectra of 72 h SA-treated cells of COSY (Figure 7.8), HMQC (Figure 7.9) and HMBC (Figure 7.10).

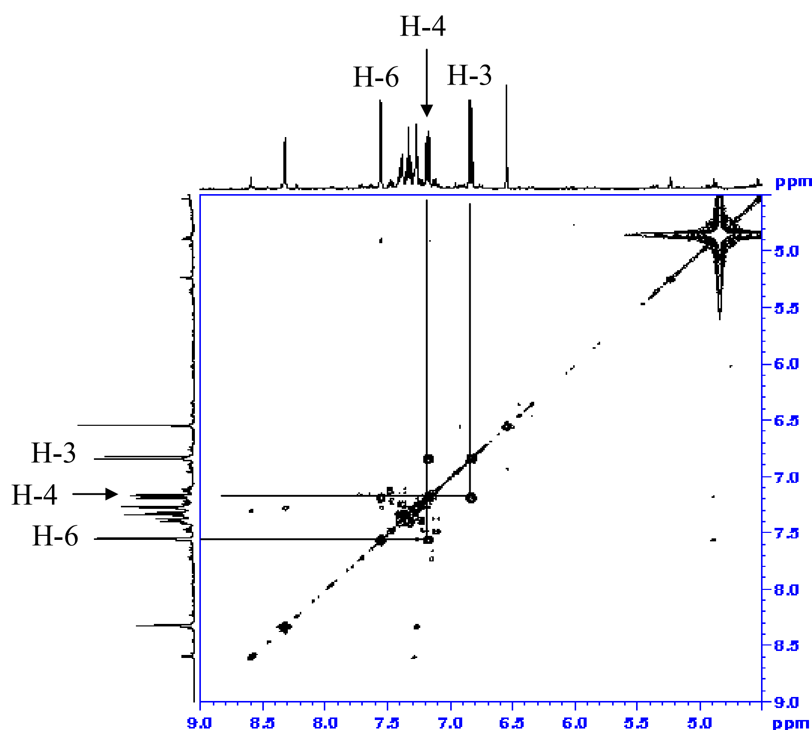

Figure 7.8. COSY spectrum (CH_3OH-d_4-KH_2PO_4 in H_2O-d_2 pH 6.0, 1 : 1) of the extract of *Catharanthus roseus* suspension cells 72 h after elicitation with sodium-salicylic acid, indicating the presence of 2,5-dihydroxybenzoic acid glucoside.

The signal at δ 4.88 (*d*, *J* = 7.5 Hz) is due to the anomeric proton (H-1') of glucose attached to the hydroxyl group of C-5 of 2,5-DHBA. The 2,5-DHBAG signals assigned by Shimoda *et al*. (2002) were found a bit shifted due to the different NMR-solvent used. This group reported the conversion of SA into gentisic acid-glucoside in *C. roseus* suspension cells, almost 100% conversion of the fed SA was observed. In our experiments the levels of 2,5-DHBAG in the cells represent only 10% of the SA fed. As media were not analyzed, we cannot exclude that the amounts of benzoic acid derivatives present in the media would complete the mass balance of the SA bioconversion. From these results there is also no direct evidence that 2,5-DHBA is formed from SA, though considering Shimoda *et al*. (2002) results one may expect that this is formed from SA by oxidation followed by glucosylation.

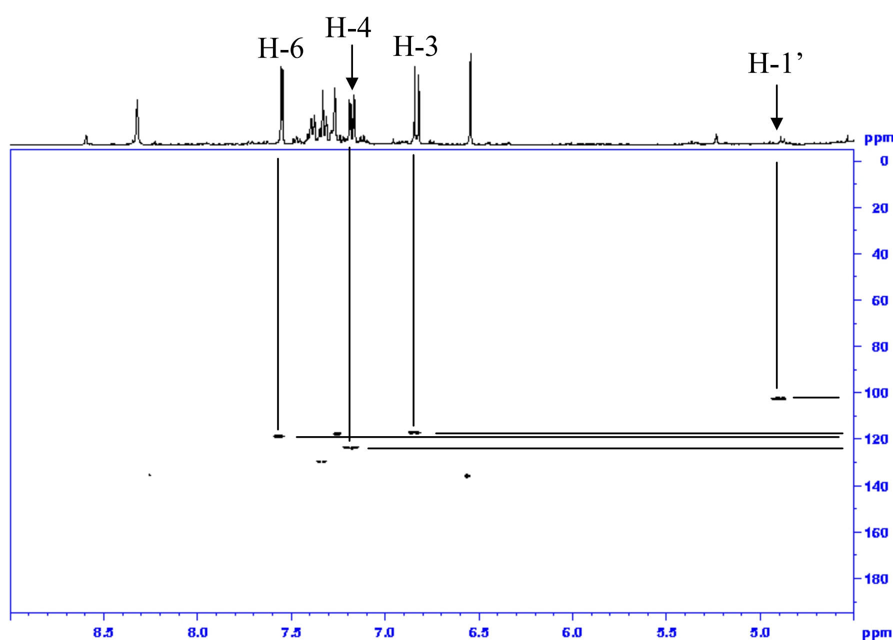

Figure 7.9. HMQC spectrum (CH_3OH-d_4-KH_2PO_4 in H_2O-d_2 pH 6.0, 1 : 1) of the extract of *Catharanthus roseus* suspension cells 72 h after elicitation with sodium-salicylic acid, indicating the presence of 2,5-dihydroxybenzoic acid glucoside.

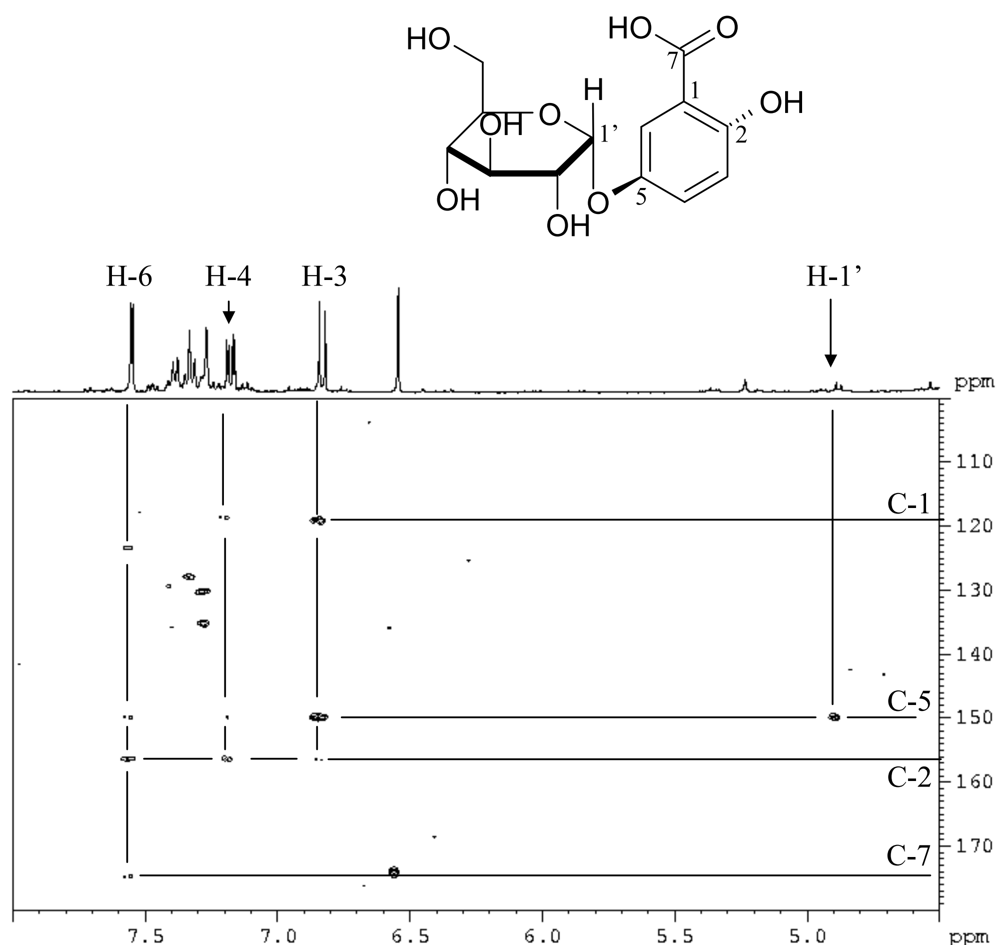

Figure 7.10. HMBC spectrum (CH_3OH-d_4-KH_2PO_4 in H_2O-d_2 pH 6.0, 1 : 1) of the extract of *Catharanthus roseus* suspension cells 72 h after elicitation with sodium-SA, indicating the presence of 2,5-dihydroxybenzoic acid glucoside (2,5-DHBAG).

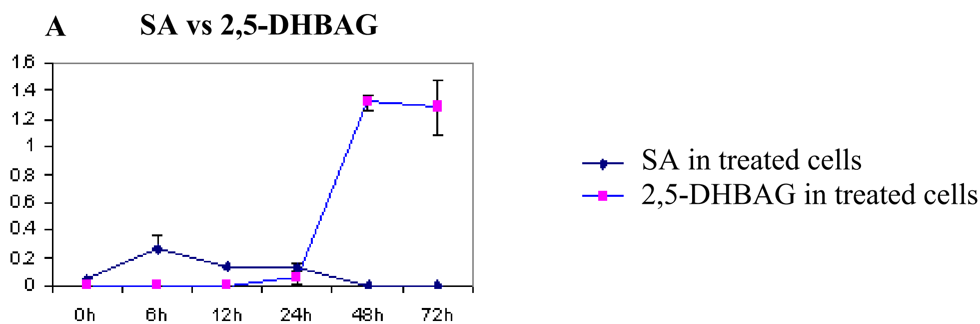

Figure 7.11. The levels of SA vs 2,5-DHBAG in the SA-treated cells of *Catharanthus roseus* in a time course (0-72 h) determined by a comparison of the peak-integrals of the compounds to the peak-integral of TSP (100%).

Figure 7.11 shows the time course of the levels of SA and its possible catabolic product 2,5-DHBAG (gentisic acid-glucoside) in the cells as compared to TSP (100%). The signals of 2,5-DHBAG appeared weakly at 24 h and strongly increased in intensity at 48 and 72 h, providing a negative effect in PC1 (Figure 7.2). Gentisic acid was also reported to be present in *C. roseus* cell cultures (Budi Muljono *et al*., 1998).

Though SA could be clearly observed in the NMR spectra of 0-24 h SA-treated cells, surprisingly SA-glucoside (SAG) could not be detected in the NMR spectra of any of the samples. The proton signals of SAG in the aromatic region are shifted to higher ppm than those of SA (data not shown), but such signals were not observed. Besides the absence of the proton signals of SAG in the aromatic region of the J-resolved NMR spectra, the HMQC and HMBC spectra of 72 h of SA-treated cells also did not show any coupling signal of SAG (Figure 7.9 - 7.10). In the previous experiments (see chapter 4) endogenous SAG was analyzed as free SA after an acid-hydrolysis using HPLC-fluorescent detection, which is much more sensitive than NMR (ng- vs µg level). Different elicitors used (SA vs *Pythium* extract) may also determine the activation of the SA-catabolic pathway.

No signals of loganic acid, secologanin (Choi *et al.*, 2004) and TIAs (such as ajmalicine, catharanthine, stemmadenine, tabersonine, vindoline, vincristine, vinblastine, anhydrovinblastine or alstonine) were observed in any of the spectra of both control- and SA-treated cells. NMR has been shown to be the method of choice for general metabolic profiling in terms of reproducibility, analysis-time and ease of sample preparation (Choi *et al*., 2004; Kim *et al*., 2006). However, for sensitivity to detect compounds present in very low levels like alkaloids in this particular *C. roseus* cell line, a more sensitive detection method than NMR (such as HPLC-DAD or -MS) might be needed. The study performed by Papon *et al*. (2005) showed that a *C. roseus* suspension cell culture fed with a combination of auxin and cytokinin increased the expression of some genes of the TIA pathway resulting in an increased-level of ajmalicine. In this study, we used the *C. roseus* A12A2 line grown in M&S medium without growth hormones that produced a much higher level of endogenous SA (detected by HPLC-fluorescence) as compared to the CRPM line grown in M&S medium with a combination of auxin and cytokinin upon elicitation with *Pythium* (Chapter 4 of this thesis). In an experiment using the same *C. roseus* line fed with

loganin and tryptamine, MeJA caused a high level of accumulation of strictosidine and ajmalicine, but SA decreased the level of ajmalicine compared to the control fed sample (El Sayed and Verpoorte, 2002). Thus, besides the sensitivity of detection, the absence of alkaloids might also be due to the *C. roseus* A12A2 cell line that has switched on other pathways than those leading to TIAs upon elicitation with SA.

Kaemferol and quercetin are common flavonoids found in plants and cell cultures. Brun *et al*. (2003) reported the presence of flavonoids in a *C. roseus* cell culture. However we could not detect the kaemferol and quercetin signals in the NMR spectra of all observations.

7.4 Conclusion

Application of ^{1}H-NMR, J-resolved ^{1}H-NMR in combination with PCA on the extracts of *C. roseus* suspension cells clearly showed altered-metabolites profiles between control cells and SA-treated cells observed during a time course of 0-72 h. SA signals already appeared weakly at 0 h and were detected clearly at 6-24 h. Sugars (glucose and sucrose) were found in increased-levels at 0-24 h in the SA-treated cells compared to the control cells (highest levels at 0 h), but depletion of sugars occurred at 48-72 h in both control- and SA-treated cells. The levels of some aliphatic amino acids and organic acids in the SA-treated cells showed two maxima at 6 h and 48 h. The fed SA had disappeared at 48 h, but at the same time gentisic acid-glucose (2,5-DHBAG) signals appeared. No loganic acid, secologanin or alkaloids were detected in the spectra. Overall the metabolic response has two phases, an immediate change of the metabolome followed by a partly return to normal and a subsequent second major change in metabolome. The two phases probably represent an immediate biochemical response, followed by a second response on gene level.