The early stress response of jasmonic acid in cell suspension cultures of Catharanthus roseus

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Chapter 6

Metabolism of exogenous jasmonic acid in cell suspension cultures of *Catharanthus roseus*

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ABSTRACT

After jasmonic acid (JA) elicitation of cell suspension cultures of *Catharanthus roseus* (L.) G. Don, derivatives such as jasmonoyl-L-isoleucine (JA-Ile), 12-hydroxyjasmonic acid (12-HOJA), 12-*O*-glucosyl-jasmonic acid (12-*O*-Glc-JA), 12-carboxyjasmonoyl-L-isoleucine (12-HOOCJA-Ile), 12-hydroxyjasmonoyl-L-isoleucine (12-HOJA-Ile) and 9,10-dihydrojasmonic acid (DHJA) accumulated in cells and growth medium shortly after addition of JA. The major products in the growth medium were JA followed by 12-HOJA, DHJA and 12-HOJA-Ile already present at 0 min. At 1440 min contents of all jasmonates (JAs) in cells reached negligible levels. The profiles over time of JA and 12-HOJA showed an inverse correlation in both cells and growth medium. A similar inverse behavior was observed for JA-Ile in cells and growth medium. The major catabolic events in *C. roseus* cells are hydroxylation, oxidation and reduction of JA, JA-Ile and 12-HOJA.

6.1 INTRODUCTION

Jasmonates (JAs) are lipid-derived messengers that regulate plant defense responses and many developmental processes. After the biosynthesis of jasmonic acid (JA) is finished in the peroxisome, the molecule epimerizes into the more stable *trans* configuration (3*R*, 7*S*)-JA (Wasternack *et al.,* 2006). Subsequently, JA may be metabolized into a number of derivatives through several known conversion pathways that may render either active, inactive or partially active metabolites (Wasternack and Song, 2017; Wasternack and Feussner, 2018) (Fig. 1). These metabolic conversions include: (1) *Hydroxylation* at C11 or C12 to 11-hydroxyjasmonic acid (11-HOJA) and 12-hydroxyjasmonic acid (12-HOJA), catalyzed by JASMONATE-INDUCED OXYGENASE 1-4 (JOX) (Caarls *et al.,* 2017); (2) *Conjugation* at C1 with the amino acids tryptamine, tryptophan, valine, leucine, tyrosine, methionine, alanine, glutamine and isoleucine. Conjugation to the biologically active (3*R,* 7*S)*-jasmonoyl-L-isoleucine (JA- Ile) (Fonseca *et al.,* 2009) is catalyzed by JASMONATE RESISTANT1 (JAR1) (Staswick *et al.,* 1992; Staswick and Tiryaki, 2004). Conjugation of *oxo*-phytodienoic acid (OPDA) to isoleucine (OPDA-Ile) was detected in *Arabidopsis* (Floková *et al.,* 2016); (3) *Reduction* of the C6 keto group yields dihydrojasmonic acid (9,10-DHJA) (Miersch *et al.,* 1989; Meyer *et al.,* 1989) and 6-HOJA; (4) *Methylation* to methyl jasmonate (MeJA) is catalyzed by JASMONIC ACID CARBOXYL METHYL TRANSFERASE (JMT) (Seo *et al.,* 2001); (5) *Glucosylation* to 1-β-glucosyl-JA (1-β-Glu-JA) and 12-*O*-β–D-glucopyranosyl-JA (JAG); (6) *Decarboxylation* at C1 yields the highly volatile *cis*-jasmone (Koch *et al.,* 1997); (7) *Conjugation* to 1-aminocyclopropane-1-carboxylic acid (JA-ACC); (8) *O-Glucosylation* of 12-HOJA to 12-*O*-glucosyl-JA (12-*O*-Glc-JA); (9) *Sulfation* of 11/12-HOJA to 11/12-*O*-sulfonyl-JA (11/12-HSO_4-JA), catalyzed by the SULFOTRANSFERASE 2a (ST2a) (Gidda *et al.,* 2003); (10) *esterification* to lasiojasmonates A-C (lasioJAs) (Andolfi *et al.,* 2014). Further modifications of JA-Ile include: (11) *Hydroxylation* to 12-hydroxyjasmonoyl-L-isoleucine (12-

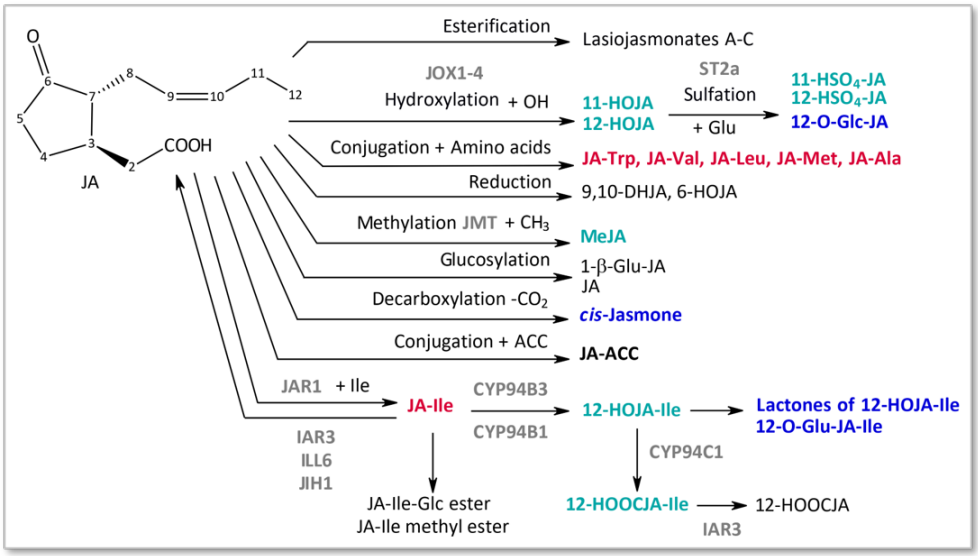

Figure 1. Metabolism of jasmonic acid (adapted from Wasternack and Feussner, 2018 and Wasternack and Strnad, 2015). Enzymes are in gray. Inactive metabolites are in green; partly active metabolites are in blue; active metabolites are in red and metabolites with unknown biological activity are in black. Metabolic conversions shown are: *esterification* to lasiojasmonates A-C; *hydroxylation* to 11-hydroxyjasmonic acid (11-HOJA) and 12-hydroxyjasmonic acid (12-HOJA), catalyzed by JASMONATE-INDUCED OXYGENASES (JOX1-4); *conjugation* to amino acids like isoleucine to JA-Ile is catalyzed by JASMONATE RESISTANT1 (JAR1) and the reverse reaction from JA-Ile to JA by the amidohydrolases IAA-ALA-RESISTENT3 (IAR3) and IAA-LEU RESISTENT-like6 (ILL6); *reduction* to 9,10-dihydrojasmonic acid (DHJA) and 6-HOJA; *methylation* to methyl jasmonate (MeJA), catalyzed by JASMONIC ACID CARBOXYL METHYL TRANSFERASE (JMT); *glucosylation* to 1-β-glucosyl-JA (1-β-Glu-JA) and 12-*O*-β–D-glucopyranosyl-JA acid (JAG); *decarboxylation* to *cis*-jasmone; conjugation to 1-aminocyclopropane-1-carboxylic acid (JA-ACC); *O*-glucosylation of 12-HOJA to 12-*O*-glucosyl-jasmonic acid (12-*O*-glucosyl-JA); *sulfation* of 11/12-HOJA to 11/12-HSO_4-JA is catalyzed by SULFOTRANSFERASE 2a (ST2a). The cytochrome P450 enzymes CYP94B3 and CYP94B1 catalyze the *hydroxylation* of JA-Ile to 12-hydroxyjasmonoyl-L-isoleucine (12-HOJA-Ile). JASMONOYL-L-ISOLEUCINE HYDROLASE 1 (JIH1) *hydrolyzes* JA-Ile to JA. CYP94C1 catalyzes the *oxidation* to 12-HOOCJA-Ile, further *deconjugated* to 12-HOOCJA by IAR3. Lactones of 12-HOJA-Ile are shown as derivatives expected to be formed although not detected yet in plants. JA-Ile can be further converted to JA-Ile methyl ester and JA-Ile-glucosyl ester (JA-Ile-Glc ester).

HOJA-Ile), catalyzed by the cytochrome P450 enzymes CYP94B3 and CYP94B1; (12) *Oxidation* of 12-HOJA-Ile to 12-carboxyjasmonoyl-L-isoleucine (12-HOOCJA-Ile), catalyzed by CYP94C1; (13) *Glucosylation* to JA-Ile-glucosyl ester (JA-Ile-Glc ester); (14) *Methylation* to JA-Ile methyl ester; (15) *Deconjugation* of 12-HOOCJA-Ile and JA-Ile, catalyzed by IAA-ALA-RESISTENT3 (IAR3) and IAA-LEU RESISTENT-like6 (ILL6) (Sánchez-Carranza *et al.,* 2016) and (16) *Hydrolyzation* to JA catalyzed by JASMONOYL-L-ISOLEUCINE HYDROLASE 1 (JIH1) (Woldemariam *et al*., 2012; 2014) (Fig. 2).

Katsir et al. (2008a) defined bioactive JAs as JA derivatives that directly promote CORONATINE INSENSITIVE 1 (COI1) interaction with one or more JASMONATE ZIM DOMAIN

(JAZ) proteins to activate gene expression. Non-bioactive JAs are either precursors or deactivated forms of bioactive JAs like 12-HOJA (Miersch *et al.,* 2008). Katsir et al. (2008b) described the following criteria to determine whether a particular compound is a bioactive signal: (i) the compound is synthesized in plant cells; (ii) the exogenous (added) compound elicits a physiological response in wild type but not in the null mutant insensitive to JAs *coi1* plants; (iii) depletion of the compound in plant tissues by genetic or pharmacological means impairs physiological responses that depend on JA; and (iv) the compound directly promotes COI1-JAZ interaction. According to Katsir et al. (2008b) of all JAs known, only JA-Ile has been shown to fulfill all four criteria. 12-*oxo*-Phytodienoic acid (OPDA), JA and MeJA only fulfill the first three criteria. The strong correlation between JA and JA-Ile suggests a reversible equilibrium of both compounds since high levels of JA are a prerequisite for JA-Ile formation and the reverse reaction from JA-Ile to JA halts the signaling pathway, thus JA availability is a controlling factor (Sánchez-Carranza *et al.,* 2016).

The biological activity of 12-HOJA and its derivatives *i.e.* 12-*O*-β-glucopyranosyl-JA and 12-HSO_4-JA, include the indirect induction of tuber formation in *Solanum tuberosum* and *Helianthus tuberosus* plants (Simko *et al*., 1996; Sato *et al*., 2009; Koda and Okazawa, 1988; Seto *et al*., 2009; Matsuura *et al.,* 1993). Moreover, a specific enantiomer of JAG is active as a leaf-closing factor in *Albizzia* and *Samanea* by binding to specific motor cells responsible for nyctinastic movements mediated by rapid potassium fluxes in a COI1-JAZ independent pathway (Nakamura *et al.,* 2011) where the stereochemistry of the glycone moiety affects its biological activity and target affinity (Ueda *et al.,* 2015). This JA derivative has also been found to play a role in male flower determination in tassels of *Zea mays* (Acosta *et al.,* 2009). 12-HOJA, 12-HOJA-Ile and 12-HOOCJA-Ile were found to accumulate within 2-5 min in wound-induced leaves of *Arabidopsis* (Glauser *et al.,* 2008a) whereas 12-*O*-glucosyl-JA, 12-HOJA and 12-HSO_4-JA accumulated late in wound-induced leaves of *S. lycopersicum*. Moreover 12-HOJA and 12-HSO_4-JA downregulated the expression of wound-inducible genes (Miersch *et al.,* 2008). The naturally occurrence of jasmine ketolactone (JKL), a lactone of 12-HOJA in *Nicotiana attenuata* leaves, suggests the possibility that the inactive 12-HOJA-Ile can be converted into active derivatives. The latter based on the fact that two synthetic macrolactones of 12-HOJA-Ile, the (3*R*, 7*R*) and the (3*S*, 7*S*) diastereomers, were able to induce nicotine accumulation in a COI1-dependent manner (Jiménez-Alemán *et al.,* 2015). Nevertheless, hydroxylation, oxidation and sulfation of JA, JA-Ile and 11/12-HOJA are regarded as the attenuation of JA and JA-Ile signaling pathways, by decreasing their biological activity and causing a partially altered or decreased gene expression of JA-related responses (Miersch *et al.,* 2008). Other metabolic conversions like the methyl esterification of JA-Ile, reduce its ability to promote COI1-JAZ interaction in *Arabidopsis* plants indicating that esterification reversibly inactivates JAs (Fonseca *et al.,* 2009). In the case of MeJA, it is biologically inactive unless it is metabolized to JA and then conjugated to JA-Ile (Tamogami *et al.,* 2008). *S. lycopersicum* plants are able to metabolize airborne MeJA into JA, JA-Ile, JA-Val, 12-HOJA and 12-

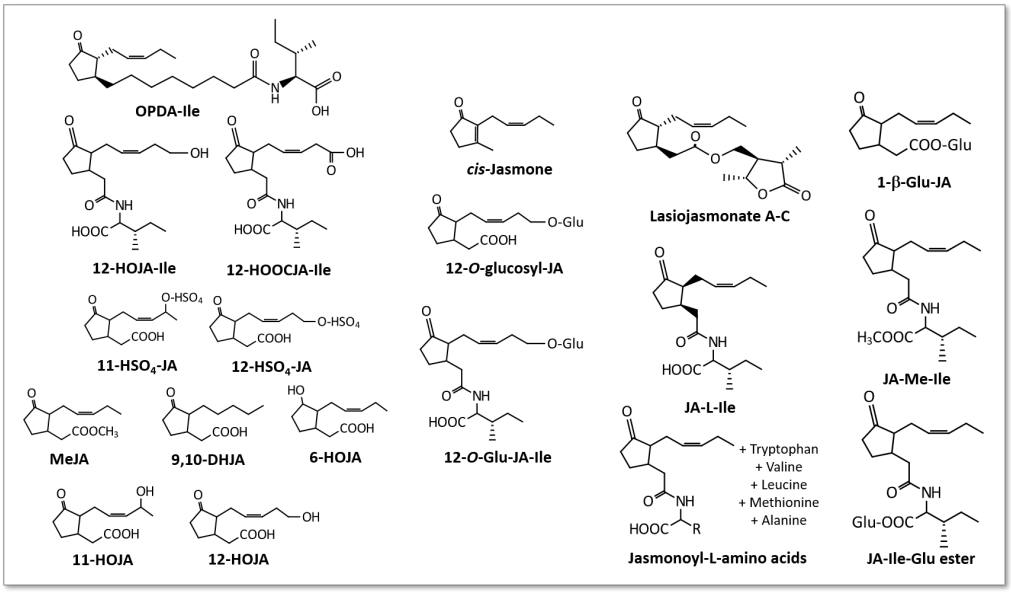

Figure 2. Some metabolic conversions of jasmonic acid, jasmonoyl-L-isoleucine (JA-Ile) and *oxo*-phytodienoic acid (OPDA). These include hydroxylation, conjugation to amino acids, oxidation, decarboxylation, methylation, reduction, sulfation, deconjugation, glucosylation, *O*-glucosylation and esterification. Biologically active jasmonates (JAs) are those conjugated to amino acids like isoleucine like JA-Ile and can be also glucosylated like 12-*O*-glucosyl-JA and 12-*O*-glucosyl-JA-Ile.

O-Glc-JA (Oki *et al.,* 2019). MeJA might regulate JA levels during fruit development in *Fragaria vesca* (Preuß *et al.,* 2014). Furthermore, glucosylation to JA/JA-Ile-Glc esters yields metabolites with unknown biological activity. Other biologically active JAs include jasmonoyl-L-tyrosine (JA-Tyr), jasmonoyl-L-valine (JA-Val), jasmonoyl-L-phenylalanine (JA-Phe), jasmonoyl-L-leucine (JA-Leu), jasmonoyl-L-alanine (JA-Ala), jasmonoyl-L-glutamine (JA-Gln) and jasmonoyl-L-tryptophan (JA-Trp), all involved in wounding and stress responses (Gapper *et al.,* 2002; Suza and Staswick, 2008; Kramell *et al.,* 1995a; 1995b; Staswick and Tiryaki, 2004; Staswick, 2009; Tamogami *et al.,* 1997). Their ability to promote interaction of *S. lycopersicum* COI1 and JAZ1/3 *in vitro* (Katsir *et al.,* 2008b) suggests that these compounds may be active signals in the wound response even though that the accumulation of JA-Ile is at least 10-fold higher. *cis*-Jasmone, one of the main components in plant volatiles, quickly accumulated in different elicited plant cell cultures (Mueller *et al.,* 1993). Although it was formerly believed to be an inactive JAs disposed through the gas phase (Koch *et al.,* 1997), it was later found to be released during insect herbivory by influencing a different signaling pathway from that of JA in *Arabidopsis* plants (Matthes *et al.,* 2010). *cis*-Jasmone has also a repellent activity against aphids (Bruce *et al.,* 2008) and induces the production of volatiles including the monoterpene (*E*)-β-ocimene, which in turn attracts parasitic insects (Birkett *et al.,* 2000).

There is quite some detailed knowledge on JA metabolism, although the steps in the inactivation of JA-Ile have been recently described. Inactivation of JA-Ile can occur via three possible ways *i.e.* either by *deconjugation* through hydrolysis of the jasmonoyl residue from the isoleucine moiety; by *oxidative inactivation* through a sequential ω-oxidation of the pentenyl site-chain of JA-Ile or by *hydrolysis* to JA. The enzymes responsible of the catalytic hydrolysis of the amide bond, IAR3 and ILL6, are located in the endoplasmic reticulum (ER) (Sánchez-Carranza *et al.,* 2016). These enzymes were identified as members of the indole-3-acetic acid (IAA) amido-hydrolase enzyme family and are essential for JA-homeostasis in *Arabidopsis* (Widemann *et al.,* 2013). On the other hand, the enzymes involved in the oxidative inactivation of JA-Ile were identified in *Arabidopsis* as belonging to the CYP94 cytochrome P450 family of enzymes and are also localized in the ER (Koo *et al.,* 2014). In *N. attenuata,* JIH1, a close homologue to IAR3, hydrolyzes JA-Ile to JA *in vitro* and its function was further confirmed in the RNA silenced *NaJIH1* gene plants where levels of JA-Ile increased significantly after herbivory (Woldemariam *et al*., 2012; 2014). The enzyme CYP94B3 is responsible for the accumulation of 12-HOJA-Ile after wounding (Koo *et al*., 2011; Kitaoka *et al*., 2011; Heitz *et al*., 2012) and can also catalyze the oxidation of JA-Ile to 12-HOOCJA-Ile, although CYP94C1 is mainly involved in this reaction (Heitz *et al*., 2012; Koo and Howe, 2012). More recently, additional enzymes such as CYP94B1 and CYP94B2 were found to inactivate JA-Ile. These two CYP94 enzymes can also catalyze the hydroxylation and oxidation of JA-Phe (Widemann *et al*., 2015), JA-Leu and JA-Val (Kitaoka *et al*., 2014). In this chapter we report on our study of the metabolic fate of exogenously applied JA; we screened cells and liquid media of cell suspension cultures of *Catharanthus roseus* (L.) G. Don to complete the picture of JA biosynthesis (Chapter 3, Chapter 5) and catabolism (present Chapter) after elicitation in our plant model system.

6.2 EXPERIMENTAL

6.2.1 Cell suspension cultures and elicitation with jasmonic acid

Cell suspension cultures of the *C. roseus* cell line CRPP were grown in 250 mL Erlenmeyer flasks containing 50 mL of Gamborg B5 medium (Gamborg *et al.,* 1968) supplemented with 30 g/L sucrose and 1.86 mg/L of 1-naphthalene acetic acid (NAA) and adjusted to pH 5.8 with 0.1 N KOH. Cell cultures were propagated on a rotary shaker (110 rpm) at 25 °C under continuous light (500-1500 lux) and were subcultured every three weeks by transferring 20 mL of the suspended cells to 50 mL of fresh medium. Four-day-old cell suspension cultures were treated with JA (7.18 µmol/flask; Sigma-Aldrich, St Louis, MO, USA) dissolved into 40% ethanol (v/v) and were harvested in quadruplicates at 0, 5, 30, 90, 360 and 1440 min after elicitation. Cells were filtered on Whatman filter paper under partial vacuum and biomass and media samples were immediately frozen in liquid nitrogen and kept at -80 °C; cells and growth media were lyophilized before any further processing.

6.2.2 Chemicals used for cell suspension cultures

The chemicals used for macro salts were: $CaCl_2$ (min. 99%), KH_2PO_4 (min. 99.5%), KNO_3 (min. 99%) and NH_4NO_3 (min. 99%) purchased from Merck (Darmstadt, Germany) and $MgSO_4$ obtained from OPG Farma (BUVA BV, Uitgeest, The Netherlands). The chemicals used for micro salts were: H_3BO_3, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, Na_2EDTA (Merck) and $FeSO_4.7H_2O$ (Brocades-ACF Groothandel NV, Maarssen, The Netherlands) dissolved into one solution and KI, $NaMoO_4.2H_2O$, $CuSO_4.5H_2O$ and $CoCl_2.6H_2O$ (Merck) dissolved into another solution to avoid insolubility problems. Thiamine-di-HCl was from Janssen Chimica (Geel, Belgium), pyridoxine-HCl was from Sigma-Aldrich Chemie (Steinheim, Germany), nicotinic acid (99.5%), glycine (99.7%) and NAA were from Merck (Darmstadt, Germany), sucrose (99.7%) and *myo*-inositol (99.7%) were from Duchefa Biochemie (Harlem, The Netherlands).

6.2.3 Extraction procedures

Approximately 100 mg of lyophilized plant material and lyophilized culture media were extracted twice with 2 mL of isopropanol, vortexed for 1 min, sonicated for 15 min and centrifuged for 15 min at 3,500 rpm at 10 °C. The extracts were dried under a gentle flow of N_2 gas, reconstituted into 1 mL of methanol-water (85:15 v/v) and subjected to SPE purification (Waters Sep-Pak C18, 1 mL, 100 mg; Milford, MA, USA), previously conditioned with 1 mL methanol and 1 mL methanol-water (85:15 v/v). Samples were loaded and eluted with 1 mL methanol-water (85:15 v/v). The solution (1 mL) was filtered (Sartorius RC 0.20 µm; Gottingen, Germany) and 1 µL was injected for UHPLC-TOF-MS analyses.

6.2.4 Quantification of jasmonates

External calibration curves of JA (Sigma-Aldrich, St Louis, MO, USA), JA-Ile (Cayman Chemical, Ann Arbor, MI, USA) and (±)-9,10-DHJA (OlChelm Ltd., Olomouc, Czech Republic) were built over different concentration levels. Working solutions were prepared in 1 mL of methanol-water (85:15 v/v). Peaks were identified based on chromatographic retention times and compared to their respective reference standards. Detection and quantification were based on peak area of each JAs. Results are shown in Table 1.

6.2.5 UHPLC-TOF-HRMS analyses

UHPLC-TOF-HRMS analyses were performed on a Micromass-LCT Premier time-of-flight spectrometer (Waters, MA, USA) with an electrospray interface and coupled with an Acquity UPLC system (Milford, MA, USA). ESI conditions: capillary voltage 2400 V, cone voltage 40 V, MCP detector voltage 2400 V, source temperature 120 °C, cone gas flow 20 L/h, desolvation gas flow 800 L/h. Detection was performed in positive and negative ion modes in the *m/z* range 100-1000 Da in a

centroid mode with a scan time of 0.5 s. For the dynamic range enhancement (DRE) lock mass, a 2 μg/mL solution of leucin-enkephalin (Sigma-Aldrich) was infused through the lock mass probe at a flow rate of 10 μg/min with a Shimadzu LC pump (LC-10ADvp, Duisburg, Germany). The separation was carried out on a Waters Acquity BEH C18 UPLC column 50 mm x 1.0 mm i.d. 1.7 μm with the following solvent system: A=0.1% (v/v) formic acid-water, B=0.1% (v/v) formic acid-acetonitrile. The flow rate was 300 μL/min using 2% of B for 4.8 min, 2-98% B in 4.9 min and holding 98% B for 6 min. All samples were analyzed once.

6.2.6 Data handling

Raw UHPLC–MS data were processed using MarkerLynx® software (Waters, Milford, MA, USA). Chromatograms for all JAs are shown in Figure 6.7.2. in the supplemental information section. Before statistical analysis, distributions were tested for normality using the Shapiro-Wilk test ($p < 0.05$). A one-way ANOVA corrected for multiple comparisons with Dunnett's test ($p < 0.05$) was applied to test significant differences in contents of each JAs compared to the same JAs at t=0 (or the earliest time point whenever a JAs was absent at t=0) in cells and in growth medium. ANOVA results are shown in Table 6.7.1 in the supplementary information section. Significant differences between JA-treated cells and JA-treated growth medium in each time point were analyzed with a two-tailed unpaired *t*-test with Welch's correction (*$p < 0.1$ and * $p < 0.05$). All statistical tests were performed with GraphPad Prism software (v. 8.4.3.686, La Jolla, CA, USA).

6.3 RESULTS AND DISCUSSION

6.3.1 Metabolism of jasmonates: hydroxylation and carboxylation

In our previous experiments, feeding cell suspension cultures of *C. roseus* with labeled JA did *not* induce the accumulation of *unlabeled* JA neither in cells nor in growth medium. Feeding cells with labeled dnOPDA showed, already after 5 min, the accumulation of *labeled* JA and *labeled* JA-Ile in cells and *labeled* JA in the growth medium after 30 min, but after 24 h no remaining labeled JAs were observed in the growth medium. Levels of labeled JA and labeled JA-Ile in cells showed a 10-fold decrease (Chapter 5). Apparently, cells are capable of a rapid production of JA, but also of a rapid catabolism and excretion. The present study aimed at mapping the catabolic and excretion pathways active in *C. roseus* cells by analyzing cells and their growth medium for JA, its catabolites and derivatives. Because JA metabolites in untreated and mock-treated cells fell below the detection level in most of the samples (data not shown), it suggests that these metabolites do not occur naturally in cells of *C. roseus* and especially, that the mock treatment did not induce their contents as it happened with fatty acids (FA) (Chapter 3). Consequently, all results shown in this chapter for JA metabolites, are the direct reflection of the metabolic fate of exogenously added JA. From 7.180 nmol/flask of JA

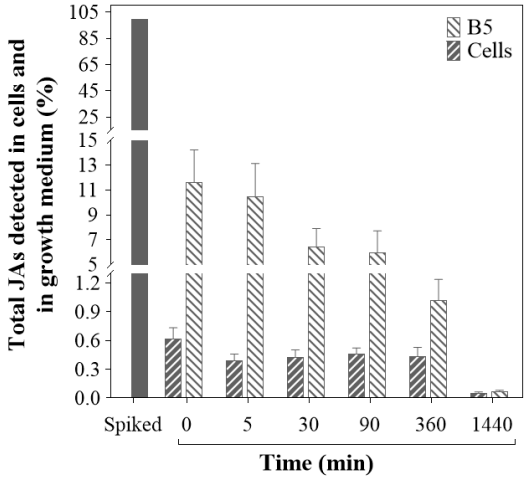

Figure 3. Percentage of total jasmonates detected in cells and growth medium (B5) of *Catharanthus roseus* measured by UHPLC-TOF-MS. Percentages were calculated from the sum of each jasmonate [nmol/flask] divided by the total amount of JA spiked [7180 nmol/flask]. Averaged data ± relative standard error of mean (SEM) of four biological replicates each analyzed once is shown. Significant differences in contents of total JAs in JA-treated cells compared to contents of total JAs in JA-treated growth medium in each time point are shown with a superscript (two-tailed unpaired *t*-test with Welch's correction, * $p < 0.1$ and ** $p < 0.05$; 0 min: t=1.116, df=4.018, p=0.3268; 5 min t=0.9674, df=3.004, p=0.4046; 30 min t=0.9983, df=5.035, p=0..3637; 90 min: t=1.022, df=5.020, p=0.3533; 360 min: t=0.5117, df=5.611, p=0.6284; 1440 min t=0.1340, df=3.539, p=0.9007).

fed to cells and growth medium, in the first time point (0 min) only 0.6 % of JAs was taken up by cells and 12.2 % remained in the growth medium (Fig. 3). From this time point onwards, JA levels in cells and growth medium decreased until JAs reached 0.04 % in both cells and medium. The profiles of JA, DHJA, 12-HOJA, 12-HOOCJA-Ile and 12-HOJA-Ile suggest that these are the major turnover metabolites in *C. roseus* cells. The possibility that the rest of the exogenous JA fed to each flask might have been converted to other JAs that were not measured such as glucosylated derivatives of JA, DHJA, 12-HOJA, 12-HOOOCJA and 12-HOOCJA-Ile and/or JA-Ile, cannot be excluded. At the time of these experiments, we only had access to 3 analytical standards *i.e.* JA, JA-Ile and DHJA, and in order to be able to compare amounts of all detected JAs in cells and growth medium, data in Figure 4 is expressed as AUC/flask for each JAs.

Of particular interest are changes of 12-HOJA in cells. This JAs was already observed at 30 min after induction reaching a maximum at 90 min (4-fold increase) and returning to negligible levels at 1440 min in cells (Fig. 4). Levels of 12-HOJA in the growth medium were relatively low at the early time points of the experiment; however, there was a 5.8-fold increase at 90 min in comparison to 0 min (Fig. 4). Contents of 12-HOJA and JA in cells had an inverse correlation supporting the idea that hydroxylation is one of the major inactivation mechanisms in *C. roseus* cells. Moreover, the presence of 12-HOJA in the medium at 0 min raises the question if extracellular oxidation also plays a role. The

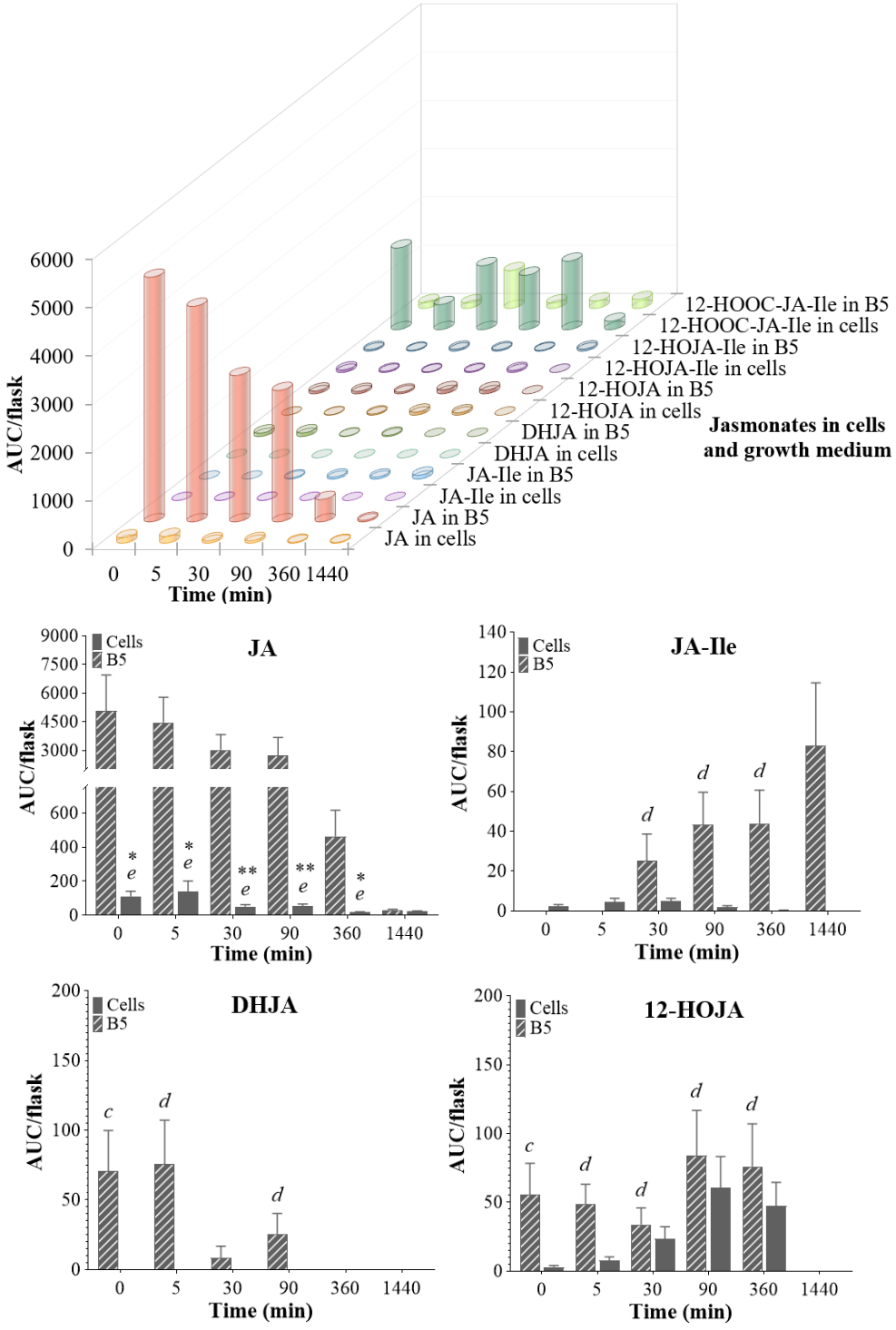

Figure 4. Continued

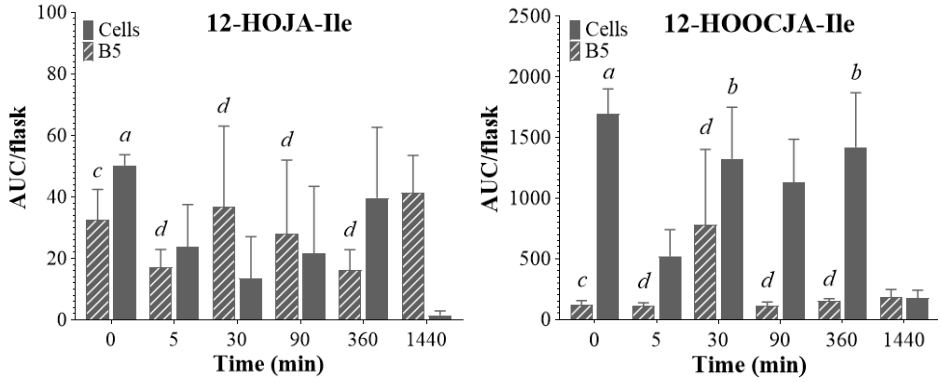

Figure 4. UHPLC-TOF-MS analysis of JA, JA-Ile, DHJA, 12-HOJA, 12-HOJA-Ile and 12-HOOCJA-Ile contents in 100 mg DW of cells and growth medium (B5) of *Catharanthus roseus* suspension cultures after JA-treatment measured by UHPLC-TOF-HRMS. Time is on the *x*-axis and area under the curve (AUC/flask) on the *y*-axis. Averaged data ± standard error of mean (SEM) of four biological replicates each analyzed once is shown. Significant differences in contents of each JAs compared to JA-treated at t=0 h and to the same or earliest time point (whenever there was no JAs present) in cells and in growth medium are marked with a superscript (one-way ANOVA with Dunnett's multiple comparison test, $p < 0.05$). Significant differences in contents of JA in JA-treated cells compared to contents of JA in JA-treated growth medium in each time point are shown with a superscript (two-tailed unpaired *t*-test with Welch's correction, * $p < 0.1$ and ** $p < 0.05$; 0 min: t=2.532, df=3.011, p=0.085; 5 min t=3.081, df=3.087, p=0.0521; 30 min t=3.623, df=3.007, p=0.036; 90 min: t=7.059, df=2.018, p=0.019; 360 min: t=2.686, df=3.005, p=0.0745; 1440 min not tested due to insufficient data).
[a] JA-treated significantly different from JA in JA-treated cells at t=0.
[b] JA-treated significantly different from JA in JA-treated cells at the same time point of observation.
[c] JA-treated significantly different from JA in JA-treated growth medium at t=0.
[d] JA-treated significantly different from JA in JA-treated growth medium at the same time point of observation.
[e] JA-treated cells significantly different from JA-treated growth medium at the same time point of observation.
No JA-Ile was detected in cells at 1440 min.
No DHJA was detected in cells at 360 and 1440 min.
No 12-HOJA was detected neither in cells nor in growth medium at 1440 min.

Table 1. UHPLC-TOF-MS analysis of JA, JA-Ile and DHJA contents in cells and growth medium of suspension cultures of *Catharanthus roseus* treated with JA (nmol/flask)

	JA		JA-Ile		DHJA	
Time (min)	**Cells**	**B5**	**Cells**	**B5**	**Cells**	**B5**
0	14.21±4.58	747.2±280.51	0.0001±0.0001	nd	nd	111.09±33.63
5	18.52±9.24	657.76±196.64	0.0007±0.0003	nd	nd	121.36±37.17
30	5.33±1.54	444.76±118.92	0.0008±0.0001	0.44±0.22	nd	15.17±0
90	5.68±1.9	531.72±63.31	nd	0.86±0.15	nd	42.89±0.16
360	0.61±0.25	63±22.86	nd	0.88±0.17	nd	nd
1440	0.94±0.51	0.92±0	nd	1.3±0.55	nd	nd

Averaged data ± standard error of mean (SEM) of four biological replicates each analyzed once by UHPLC-TOF-HRMS is shown.
B5: growth media, nd: not detected.

presence of a compound with a matching exact mass to that of 12-*O*-Glc-JA was noticed only in JA-treated cells in increasing amounts at 90-1440 min (data not shown), although its unambiguous

identification remains to be confirmed. Swiatek et al. (2004) reported similar results with tobacco BY-2 cells, where 2 h after feeding with JA and MeJA, the hydroxylated JAs 11-HOJA- and 12-HOJA were present, particularly in the liquid growth medium along with jasmonoyl-*O*-1-β-D-glucose, JA, hydroxyjasmonoyl-1-β-glucose and jasmonoyl-1-β-gentiobiose. Xia and Zenk (1993) fed cell suspension cultures of *Eschscholtzia californica* with JA and concluded that the major inactivation mechanisms were hydroxylation followed by glucosylation after finding 11-HOJA and jasmonoyl-1-*O*-β-D-glucoside along with DHJA. The same hydroxylated JAs in their glucosylated form were found in potato leaflets (Matsuura *et al.,* 2001). In wounded leaves of tobacco, the addition of JA resulted in the accumulation of JA, JA-Ile, 12-HOJA and 12-HOJA-glucoside in systemic leaves 2 h after treatment (Sato *et al.,* 2009).

DHJA was detected only in JA-treated growth medium, it was already present at 0 min, peaking at 5 min and not detected after 360 min (Fig. 4 and Table 1). DHJA had a 24-fold decrease at 30 min and a 4.2-fold decrease at 90 min, both when compared to values at 5 min. It is noteworthy that levels of DHJA at 0 min were higher than those of 12-HOJA in the growth medium at the same time point, but not higher than JA in the growth medium, suggesting an extracellular reaction of JA or with a component from the liquid growth medium. JA-Ile and JA also had an inverse behavior where contents of JA in cells had a 15.6-fold increase at 30 min in comparison to 0 min but then decreased to reach a non-detectable level at 90 min whereas JA-Ile in the growth medium showed an increase during the whole experiment, reaching the highest value at 1440 min (Fig. 4 and Table 1). This observation suggests that JA-Ile formed in the cells was excreted to the growth medium after 30 min. Oxidized and hydroxylated derivatives of JA-Ile 12-HOOCJA-Ile and 12-HOJA-Ile, were observed in cells and growth medium in our experiments. 12-HOOCJA-Ile was more abundant in cells than in the growth medium. Levels were similar throughout all the experiment except at 5 min and 1440 min (Fig. 4). Levels in the growth medium varied less if compared to cells. Levels of 12-HOJA-Ile were at least 20 times less than 12-HOOCJA-Ile throughout the experiment. 12-HOJA-Ile was also more abundant in cells at 0 min and 360 min decreasing to minimum levels at 1440 min (Fig. 4). Contents of 12-HOJA-Ile in growth medium remained similar throughout the experiment. However, because of their very low levels, their quantification was not possible. These carboxylated and hydroxylated derivatives were found to be formed in wounded leaves of *A. thaliana* 2-5 min after induction (Glauser *et al.,* 2008b) and are regarded as inactivated forms of JA-Ile as they are unable to promote *in vitro* JAZ-COI1 co-receptor assembly (Aubert *et al.,* 2015; Koo *et al.,* 2011).

Levels of JA in JA-treated cells reached their maximum at 5 min with a 1.3-fold increase in comparison to JA-treated cells at 0 min (Fig. 4 and Table 1). This observation agrees with our previous results, the accumulation of labeled JA after feeding with labeled dnOPDA peaking at 5 min (Chapter 5). In contrast, levels of JA in the growth medium slowly decreased over time reaching negligible levels after 360 min (Fig. 4 and Table 1). Furthermore, differences in contents of JA between the growth

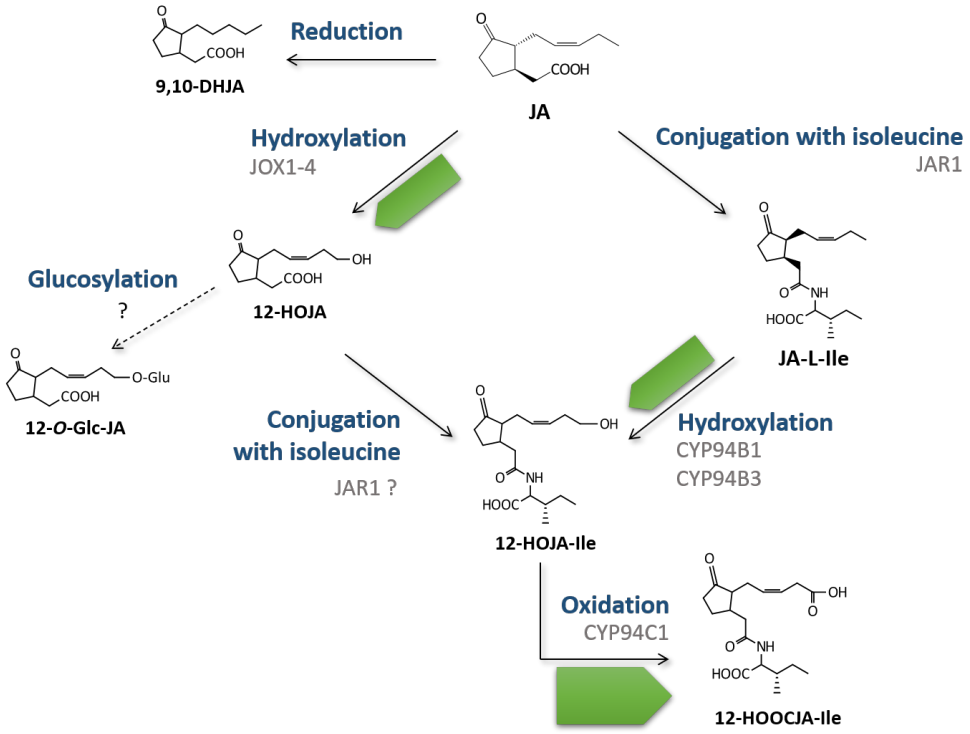

Figure 5. Model of the metabolic pathways of jasmonic acid found in cells and growth medium of *Catharanthus roseus* suspension cultures after JA-treatment measured by UHPLC-TOF-HRMS. Thickness of green arrows denote major conversions and thinner green arrows show minor ones. Dashed arrows show potential conversions of 12-hydroxyjasmonic acid (12-HOJA). Upon feeding cells with jasmonic acid (JA), the three main metabolic pathways were hydroxylation to 12-HOJA possibly catalyzed by JASMONATE-INDUCED OXYGENASES (JOX1-4) in *C. roseus* cells, conjugation with isoleucine to jasmonoyl-L-isoleucine (JA-L-Ile), catalyzed by JASMONATE RESISTANT1 (JAR1) and reduction to 9,10-dihydrojasmonic acid (DHJA). Subsequently, JA-Ile undergoes hydroxylation to 12-hydroxyjasmonoyl-L-isoleucine (12-HOJA-Ile) possibly catalyzed by the CYP94B1 and CYP94B3 enzymes in *C. roseus* cells and lastly, oxidation to 12-carboxyjasmonoyl-L-isoleucine (12-HOOCJA-Ile), possibly catalyzed by CYP94C1. We hypothesize if 12-HOJA could have been conjugated to 12-HOJA-Ile and further carboxylated to 12-HOOCJA-Ile or glucosylated to 12-*O*-glucosyl-JA (12-*O*-Glc-JA).

medium and cells were significant in all time points except for 1440 min (Fig. 5) suggesting the uptake of JA by cells from the growth medium rather than the *de novo* biosynthesis of JA.

Previous feeding experiments using cell suspension cultures of *C. roseus* have described hydroxylation and/or glucosylation events of exogenously added metabolites such as the conversion of exogenously fed Δ^9-tetrahydrocannabinol (Δ^9-THC) into different hydroxylated and glucosylated derivatives along with cannabinol (CBN) and its glycosylated derivative (Akhtar *et al.*, 2015); the accumulation of arbutin (hydroquinone-β-D-glucopyranoside) from exogenous hydroquinone (Inomata *et al.,* 1991); the hydroxylation of salicylic acid (SA) into 2,5-dihydroxybenzoic acid followed by its glycosylation of the hydroxyl group at C-5 (Mustafa *et al.,* 2009); the formation of desacetylcinobufagin

16-*O*-β-D-glucoside, 3-*epi*-desacetylcinobufagin 16-*O*-β-D-glucoside, 3-*oxo*-desacetylcinobufagin 16-*O*-β-D-glucoside and cinobufagin 3-*O*-β-D-glucoside from exogenously fed cinobufagin (Ye *et al.,* 2002): the conversion to 12β,13α-dihydroxytriptonide from exogenous triptonide (Ning *et al.,* 2004): the glucosylation of exogenously fed vanillin into glucovanillin (Yuana *et al.,* 2002) and vanillyl alcohol along with its benzyl and phenyl glucoside (Sommer *et al.,* 1997) and 4-formyl-2-methoxyphenyl-*O*-β-D-glucopyranoside in cell suspension cultures of *Coffea arabica* (Kometani *et al.,* 1993).

Glucosylation *in vitro* is a characteristic transformation in plant cells that is position-specific, that is at the β-linkage at C1 of the glucose moiety (Kometani *et al.,* 1993). This structural modification is an important regulation point in homeostasis because by changing the solubility of otherwise water-insoluble compounds, it inactivates them and facilitates their transport as it has been proven for the glucosides of salicylic acid, benzoic acid (Wasternack and Hause, 2013) and abscisic acid (Piotrowska and Bajguz, 2011). Glucosylation of JAs has been reported in wound-induction experiments in *S. lycopersicum* (Matsuura *et al.,* 2012; Sato *et al.,* 2011; Miersch and Wasternack, 2000) and *S. tuberosum* (Yoshihara *et al.,* 1996; Matsuura *et al.,* 2001; Matsuura and Yoshihara, 2003; Miersch *et al.,* 2008) and *in vitro* in cell suspension cultures of *S. peruvianum* (Schwarzkopf and Miersch, 1992). Together with hydroxylation, these further reactions might be part of a signal attenuation mechanism (Miersch *et al.,* 2008; Kitaoka *et al.,* 2014). The fact that particular derivatives of JA, *e.g.* 12-HOJA, 12-HSO_4-JA and 12-*O*-Glc-JA, occur in higher concentrations than JA in intact tissues, supports an inactivation function for these compounds (Miersch *et al.,* 2008).

The current understanding on phytohormone metabolism is still incomplete, while amino acid conjugation is crucial for activation and hence for the signaling pathway, other reversible modifications through *e.g.* esterification and glucosylation may be important for transport or as temporary form of storage, from which the active forms can be rapidly released by hydrolysis. The methylation of JA yields the volatile MeJA, which could be a messenger between plants and can more rapidly diffuse in leaves via the gas phase rather than via transport in the liquid phase. Hydroxylation and oxidation are irreversible inactivation events that at the end lead to signal attenuation. Considering the fast occurrence of metabolites in the medium extracellular conversions may occur. In addition, transport seems to play an important role, considering the high JA levels at 0 min in the cells. The catabolic enzymes seem to be constitutively expressed, regarding their immediate (0 min) activity.

6.4 CONCLUSIONS

In Chapter 5 we showed that feeding cells with *d5*-JA did not induce the accumulation of endogenous JA in cell suspensions of *C. roseus*. In agreement with that observation, JA was found to go down to basal levels within 1440 min. Here we show that the gradual decrease in JA levels in cells and the growth medium has an inverse correlation with levels of 12-HOJA. A similar pattern was found in JA-

Ile, while levels in cells increased within 30 min and went down to basal levels afterwards, in growth medium, levels of JA-Ile were only found after 30 min. Other metabolites like 12-HOJA-Ile and 12-HOOCJA-Ile were found mostly in cells although their presence in the growth medium points to their excretion. The unambiguous identification of 12-*O*-Glc-JA is to be confirmed. The 12-hydroxylation, 12-oxidation and reduction to DHJA seem to be the main catabolites of JA in cell suspension cultures of *C. roseus* (Fig. 4). These JAs were neither in untreated nor mock-treated cells present, which strongly suggests that these derivatives are the result of the metabolic fate of exogenously fed JA. Moreover, the sole presence of these metabolites shows their immediate conversion suggesting that the enzymes involved are constitutively present and active in cells of *C. roseus*. Based on the type of metabolites found, we expect the presence of the enzymes CYP94B3, CYP94B1, CYP94C1, JOX1-4 and JAR1, which would need further investigation. Finally, whereas JA, 12-HOJA, JA-Ile and DHJA were clearly detected as substrate and initial derivatives in this experiment, further oxidation, conjugation, hydroxylation and/or glucosylation of the various metabolites are most probably involved in cell suspensions of *C. roseus*, although this needs further testing.

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6.7 SUPPLEMENTAL INFORMATION

Table 6.7.1. ANOVA results for each test performed for each jasmonate in JA-treated cells and growth medium compared to JA in JA-treated cells and growth medium of suspension cultures of *Catharanthus roseus*

			Time (min)			
	0	**5**	**30**	**90**	**360**	**1440**
JAs Cells	$F_{(3,10)} = 16.56$ $p=0.0003$	$F_{(3,9)} = 1.837$ $p=0.2108$	$F_{(4,10)} = 4.593$ $p=0.0231$	$F_{(3,8)} = 2.508$ $p=0.1327$	$F_{(3,10)} = 6.557$ $p=0.0100$	$F_{(2,5)} = 1.522$ $p=0.3046$
JAs B5	$F_{(4,12)} = 5.083$ $p=0.0125$	$F_{(4,12)} = 7.835$ $p=0.0024$	$F_{(5,10)} = 6.744$ $p=0.0054$	$F_{(5,11)} = 46.33$ $p<0.0001$	$F_{(4,12)} = 5.238$ $p=0.0112$	$F_{(3,9)} = 2.187$ $p=0.1593$

JAs: jasmonates; B5: growth medium.

Figure 6.7.2. UHPLC profiles of 12-HOJA, 12-HOJA-Ile, 12-HOOC-JA-Ile, JA and DHJA detected in cells and/or growth medium of cell suspension cultures of *Catharanthus roseus.*

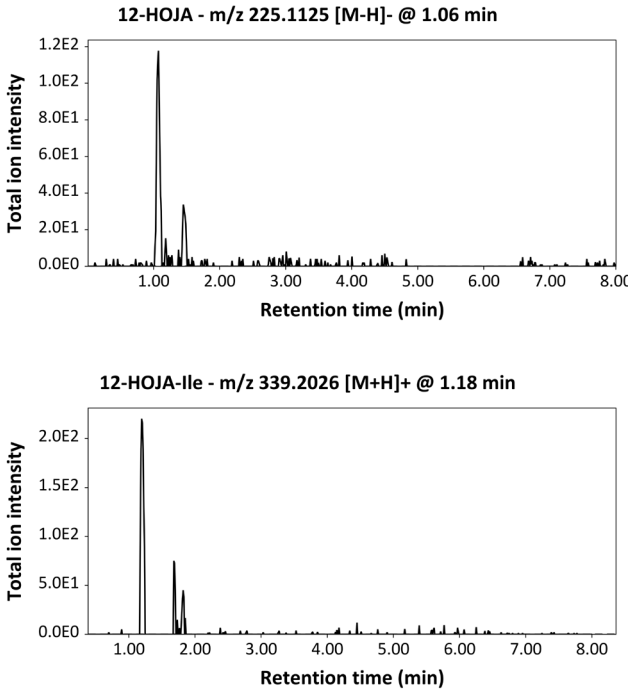
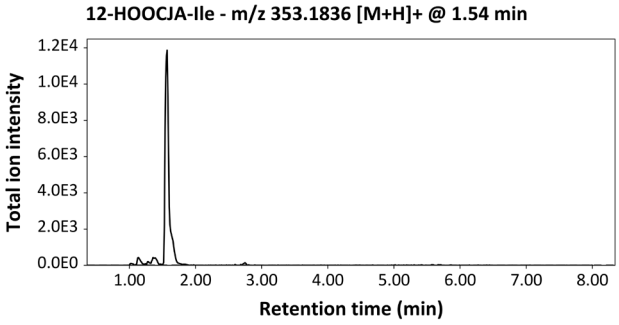
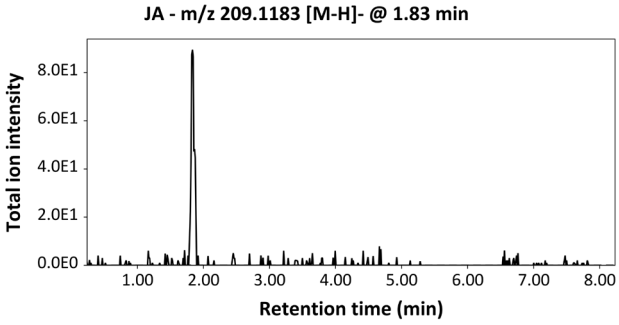
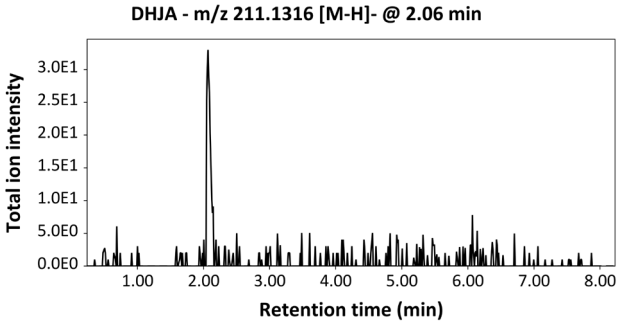
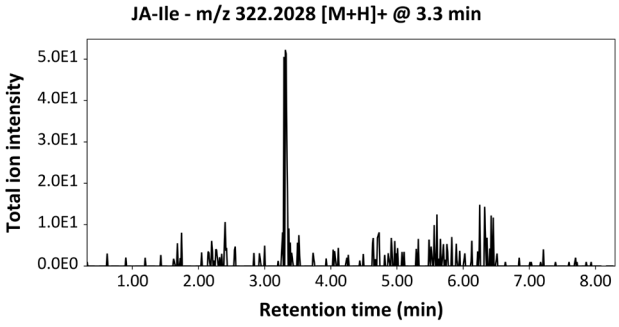


Figure 6.7.2. Continued


12-HOOCJA-Ile - m/z 353.1836 [M+H]+ @ 1.54 min
Total ion intensity
1.2E4
1.0E4
8.0E3
6.0E3
4.0E3
2.0E3
0.0E0
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Retention time (min)


JA - m/z 209.1183 [M-H]- @ 1.83 min
Total ion intensity
8.0E1
6.0E1
4.0E1
2.0E1
0.0E0
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Retention time (min)


DHJA - m/z 211.1316 [M-H]- @ 2.06 min
Total ion intensity
3.0E1
2.5E1
2.0E1
1.5E1
1.0E1
5.0E0
0.0E0
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Retention time (min)


JA-Ile - m/z 322.2028 [M+H]+ @ 3.3 min
Total ion intensity
5.0E1
4.0E1
3.0E1
2.0E1
1.0E1
0.0E0
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Retention time (min)