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

Goldhaber Pasillas, G.D.

Chapter 4

Terpenoid indole alkaloid profiles of cell suspension cultures of *Catharanthus roseus*

Goldhaber-Pasillas GD[1], Marti G[2], Wolfender JL[2], Verpoorte R[1]

[1]Natural Products Laboratory, Institute of Biology Leiden, Sylvius Laboratory, Leiden University, Sylviusweg 72, 2333BE, Leiden, The Netherlands

[2]Phytochemistry and Bioactive Natural Products, School of Pharmaceutical Sciences, University of Geneva and University of Lausanne, CMU – Rue Michel-Servet 1, 1206 Geneva, Switzerland

ABSTRACT

Cell suspension cultures of the medicinal plant *Catharanthus roseus* (L.) G. Don accumulate a wide variety of terpenoid indole alkaloids (TIA). The cell line (CRPP) used for this study accumulated 10 different TIA *e.g.* catharanthine, tabersonine, serpentine and 7 different α-methylene-indoline alkaloids (α-TIA-1-7) along with the precursors loganic acid and strictosidine upon elicitation with jasmonic acid (JA). The aim of this study was to integrate the knowledge of the fast JA-stress response with later events like the accumulation of TIA in *C. roseus.* Treatment of cells with JA resulted in an increased accumulation for catharanthine, α-TIA-1, serpentine and tabersonine and a decrease of loganic acid, 1440 min after induction when compared to JA-treated cells at 0 min or to untreated cells at the same time point of observation. Strictosidine levels were much higher than all other TIA and were more or less the same during the experiment, only at the last time point it dropped considerably if compared to t=0. Similarly, untreated and mock-treated cells showed a similar drop in strictosidine levels at 1440 min. Catharanthine did not show major differences between the first and the last time point, but at 90 and 360 min the levels were considerably lower for all three samples, with no apparent reason. Levels of loganic acid showed a decrease throughout the experiment for all samples. All these observations are in agreement with our previous results where the accumulation of TIA fit into the late stress response, which depends on the signaling pathway mediated by JA that leads to the expression of genes involved in the biosynthesis of TIA.

4.1 INTRODUCTION

The terpenoid indole alkaloids (TIA) are a group of about 2000 compounds naturally occurring amongst members of the botanical families Apocynaceae, Loganiaceae, Nyssaceae, Rubiaceae and Icacinaceae (O'Connor and Maresh, 2006; Zhang *et al.,* 2018a). Most of these TIA have specific roles in plant defense as antimicrobials, insect deterrents (Luijendijk *et al.,* 1996; Roepke *et al.,* 2010), antifungal (Guéritte *et al.,* 1983) and there is a large number of biologically active agents used in pharmacology such as the antineoplastic dimeric alkaloids vinblastine, vincristine, anhydrovinblastine from *Catharanthus roseus* and camptothecine from *Camptotheca acuminata*, the rat poison strychnine from *Strychnos nux-vomica*, the antihypertensive serpentine from *C. roseus*, the antiarrhythmic ajmalicine from *C. roseus* and *Rauwolfia serpentina*, the stimulant yohimbine from *Pausinystalia yohimbe* and *R. serpentina* and the antimalarial quinine from *Cinchona ledgeriana* to name a few (Kutchan, 1995; van der Heijden *et al.,* 2004; O'Connor and Maresh, 2006). Of particular interest is *C. roseus* (Apocynaceae), which is one of the most studied medicinal plants since it accumulates over 130 different TIA such as vincristine and vinblastine effectively used for some 40 years against different types of cancer (van der Heijden *et al.,* 2004). The amounts of the dimeric TIA in *C. roseus* leaves are very low (around 0.0005 % DW) and consequently difficult to isolate from a mixture of many

compounds with very similar chemical and physical properties (Scott, 1970). Because of these difficulties, tremendous efforts have been focused on their extraction, isolation, separation and structural elucidation using new technologies such as the use of ionic liquids (IL), supercritical fluid extraction (SFE) and molecularly imprinted polymers (MIP) besides the long-known capillary electrophoresis (CE), high-speed counter current chromatography (HSCCC), quantitative nuclear magnetic resonance (qNMR), gas chromatography coupled to mass spectrometry (GC-MS) and ultra high-pressure liquid chromatography coupled to mass spectrometry (UHPLC-MS) (Goldhaber-Pasillas *et al.,* 2013).

Owing to their commercial importance, many efforts to produce them by means of plant cell cultures have been reported (De Luca and St-Pierre, 2000; van der Heijden *et al.*, 2004) such as scale-up in bioreactors (Zhao and Verpoorte, 2007), precursor feeding experiments (Contin *et al.,* 1999; Moreno *et al.,* 1993; Whitmer *et al.,* 2002; El-Sayed and Verpoorte, 2002; Arvy *et al.,* 1994), media optimization (Contin *et al.,* 1998; Smith *et al.,* 1987a) and genetic modifications with biosynthetic genes (Goddijn *et al.,* 1995; Hughes *et al.,* 2004) and transcription factors (Peebles *et al.,* 2009a; 2009b) such as the octadecanoid-responsive *Catharanthus* AP2-domain ORCA1, ORCA2 and ORCA3 (Memelink *et al.,* 2001; Menke *et al.,* 1999a; Li *et al.,* 2013).

Cell suspension cultures of *C. roseus* are able to accumulate catharanthine and tabersonine (Verpoorte *et al.,* 1993) and to transform tabersonine in 16-methoxytabersonine (St-Pierre and De Luca, 1995) but they do not have the enzymatic activity necessary for vindoline biosynthesis (Vázquez-Flota and De Luca, 1998), which only occurs in plastids in photosynthetically active cells. The major problem is the lack of differentiation of cell cultures (Facchini and De Luca, 2008) since the many steps involved are tightly regulated and the expression of biosynthetic genes is cell-, tissue-, development- and environment-specific and also affected by external biotic and abiotic factors (Leonard *et al.,* 2009; De Luca and St-Pierre, 2000).

4.1.1 Biosynthesis of terpenoid indole alkaloids in *Catharanthus roseus*

Terpenoid indole alkaloids are derived from two primary metabolic pathways: the shikimate and the monoterpenoid (also known to be synthesized from 2-*C*-methyl-D-erythritol 4-phosphate (MEP)) pathways that require coordination of the amounts of precursors supplied by both pathways. TIA consist of an indole moiety provided by tryptamine, derived from the amino acid tryptophan and a monoterpenoid component derived from the iridoid glucoside secologanin (Facchini, 2001). The secologanin biosynthetic pathway starts in the vascular cells and/or the epidermal cells with the condensation of glyceraldehyde-3-phosphate and pyruvate to yield 1-deoxy-D-xylulose-5-phosphate catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS) (Chahed *et al.,* 2000). Next, the condensation between the isomers dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) is catalyzed by geranyl diphosphate synthase (GPPS) leading to geranyl diphosphate

(Chatzivasileiou *et al.,* 2019). Geraniol is formed from geranyl diphosphate by the enzyme geraniol synthase (GES), and through multiple enzymatic reactions that include oxidation, reduction, glycosylation and methylation, the end product is the formation of secologanin (Fig. 1). This iridoid is coupled to tryptamine, yielding strictosidine, the central precursor for all TIA. The first step in the iridoid pathway is the enzyme geraniol 10-hydroxylase/8-oxidase (G8O) which is found in the internal phloem associated parenchyma (IPAP) cells. The encoding gene was first identified by Collu *et al.* (2001). The last two steps, the enzymes loganic acid *O*-methyltransferase (LAMT) and secologanin synthase (SLS) take place in the epidermal cells (Irmler *et al.,* 2000) (Fig. 1). More recently, two additional enzymes were characterized in the secologanin pathway *i.e.* iridoid synthase (IS) (Geu-Flores *et al.,* 2012) and GES (Simkin *et al.,* 2013) (Fig. 1). The full iridoid pathway was eventually elucidated on the level of genome, transcriptome, proteome and metabolome and the complete pathway was successfully expressed in *Nicotiana benthamiana* by Miettinen *et al*. (2014). Some reviews deal with the TIA biosynthesis and regulation (De Luca *et al.,* 2014; Pan *et al.,* 2016), compartmentation and logistics (Courdavault *et al.,* 2014), metabolic engineering (Kellner *et al.,* 2015; Pan *et al.,* 2012; Thamm *et al.,* 2016) and transport (Roytrakul and Verpoorte, 2007). Recently, Qu *et al.* (2018) reported new steps in the catharanthine pathway.

The biosynthesis of all TIA starts with strictosidine which is formed through the condensation of secologanin and tryptamine in the upper and lower epidermis. Tryptamine derives from the shikimate pathway where anthranilate synthase (AS) commits chorismate to anthranilate yielding tryptophan. Decarboxylation of tryptophan to tryptamine is catalyzed by tryptophan decarboxylase (TDC). The condensation of secologanin and tryptamine is catalyzed by strictosidine synthase (STR) and takes place in the cell vacuole. Seven isoforms of STR have been identified in *C. roseus* (de Waal *et al.,* 1995) that are possibly subject to post-translational modifications as they are encoded by a single gene (Pasquali *et al.,* 1999). The next step is catalyzed by strictosidine β-glucosidase (SGD) which yields a highly reactive opened-ring dialdehyde, which through the formation of a carbinolamine between one of the aldehyde groups with the NH_2 group yields cathenamine. Different rearrangements of cathenamine lead to a large structural diversity of TIA skeletons *e.g.* strychnos-, aspidosperma-, corynanthe-, bisindole-, iboga-, quinoline- and ajmalan-type (Geerlings *et al.,* 2000; Leonard, 1999). The reduction of cathenamine by cathenamine reductase (CR) leads to the formation of ajmalicine that can be further oxidized to serpentine in the vacuole by a class III peroxidases (Blom *et al.,* 1991; Sierra *et al.,* 1989; Sottomayor *et al*., 2004). Through epimerization of cathenamine, tetrahydroalstonine is formed by reduction of the enamine/carbinolamine group. The enzyme tetrahydroalstonine synthase (THAS) is thought to be involved in this reaction (Qu *et al.,* 2018; Stavrinides *et al.,* 2015). This compound can be oxidized to yield alstonine. The biosynthetic steps starting from strictosidine leading to tabersonine on one hand and to catharanthine on the other one, are not fully characterized. The biosynthesis of both TIA is believed to occur in the epidermal cells where catharanthine is exported via

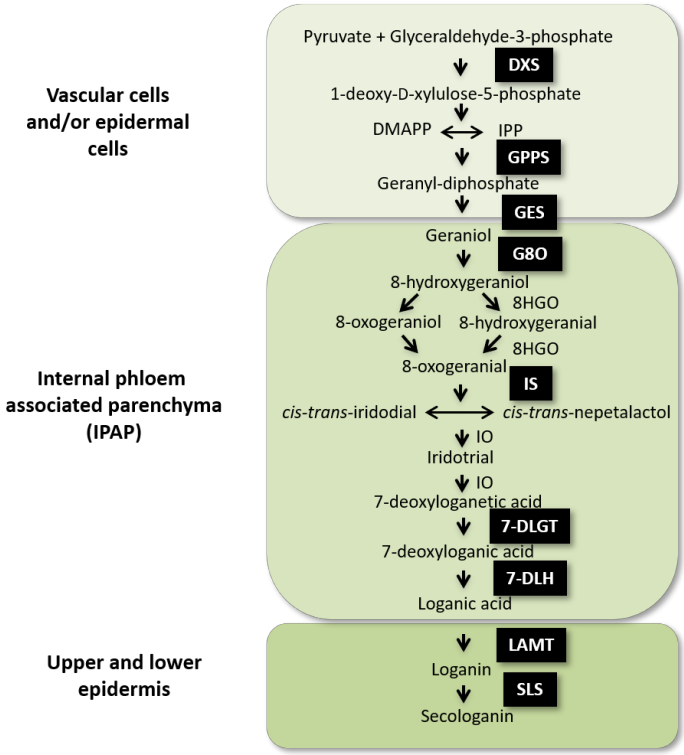

Figure 1. Biosynthetic pathway of TIA in *Catharanthus roseus* (adapted from Murata *et al.,* 2008; Liu *et al.,* 2017; Miettinen *et al.,* 2014). Framed consecutive reactions indicate localization in the plant. Genes previously reported to be regulated by ORCA3 and ORCA2, both induced by jasmonic acid (JA), are indicated in black boxes. Abbreviations for enzymes, proteins and/or products listed in sequential order are: DXS: 1-deoxy-D-xylulose-5-phosphate synthase; DMAPP: dimethylallyl diphosphate; IPP: isopentenyl diphosphate; GPPS: geranyl diphosphate synthase: GES: geraniol synthase occurring in vascular cells and/or epidermal cells; G8O: geraniol 8-oxidase; 8HGO: 8-hydroxygeraniol oxidoreductase; IS: iridoid synthase; IO: iridoid oxidase; 7-DLGT: 7-deoxyloganetic acid glucosyltransferase; 7-DLH: 7-deoxyloganic acid hydroxylase all secoiridoid pathway occurs in the internal phloem associated parenchyma (IPAP) cells and LAMT: *S*-adenosyl-L-methionine:loganic acid methyltransferase and SLS: secologanin synthase occurring in epidermal cells.

the ATP-binding cassette (ABC) transporter CrTPY2 to the leaf surface (Yu and De Luca, 2013) and tabersonine is transported to parenchymal cells. The steps that follow from tabersonine to vindoline are light dependent and need cellular differentiation (Vázquez-Flota *et al.,* 2002; De Luca and St-Pierre, 2000). The biosynthetic steps towards vindoline start with the hydroxylation of tabersonine at the C16 position catalyzed by tabersonine 16-hydroxylase (T16H) that is subsequently converted to 16-methoxytabersonine by 16-hydroxytabersonine-16-*O*-methyltransferase (16OMT) further oxidized to 16-methoxy-2,3-dihydrotabersonine by an unknown hydroxylase (St-Pierre and De Luca, 1995) (Fig. 2). The conversion to desacetoxyvindoline is catalyzed by *N*-methyltransferase 16-methoxy-2,3-dihydro-3-hydroxytabersonine (NMT) and has only been detected in differentiated plants (Dethier and

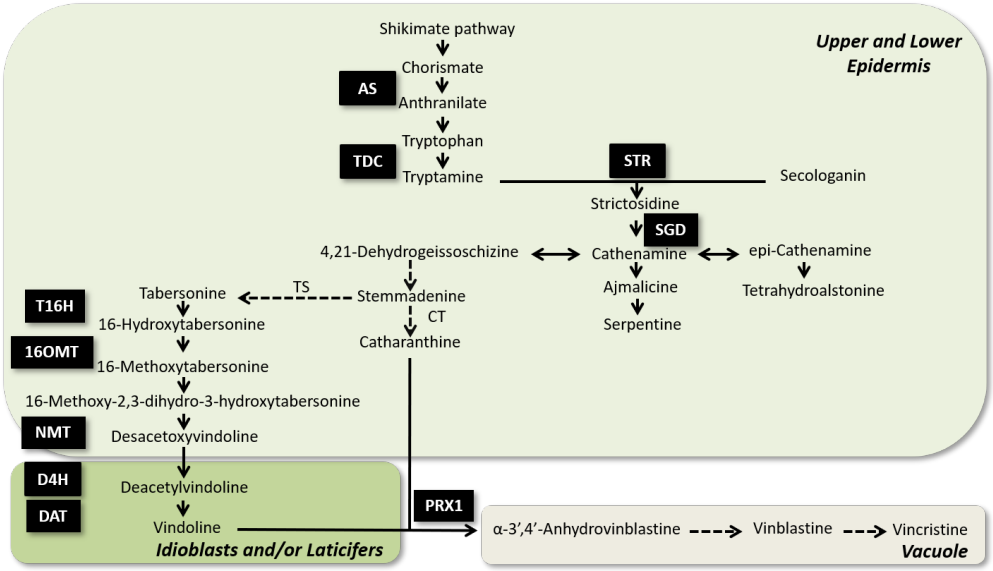

Figure 2. Biosynthetic pathway of TIA in *Catharanthus roseus* (adapted from Liu *et al.,* 2017; De Luca *et al.,* 2014). Broken arrows indicate multiple enzymatic reactions. Genes previously reported to be regulated by ORCA3 and ORCA2, both induced by jasmonic acid (JA), are indicated in black boxes. Abbreviations for enzymes and/or proteins listed in sequential order are: AS: anthranilate synthase; TDC: tryptophan decarboxylase; STR: strictosidine synthase; SGD: strictosidine β-D-glucosidase; TS: tabersonine synthase; CT: catharanthine synthase; T16H: tabersonine 16-hydroxylase; 16OMT: 16-hydroxytabersonine-16-*O*-methyltransferase occurring at the upper and lower epidermis; NMT: 16-methoxy-2,3-dihydro-3-hydroxytabersonine occurs in the epidermis; D4H: desacetoxyvindoline 4-hydrolase; DAT: acetyl-CoA:4-*O*-deacetylvindoline 4-*O*-acetyltransferase and PRX1: peroxidase 1 occurring in the idioblasts and/or laticifers in the mesophyll.

De Luca, 1993). The last two steps towards vindoline biosynthesis are catalyzed by the 2-oxoglutarate-dependent dioxygenase desacetoxyvindoline 4-hydrolase (D4H) (Vázquez-Flota and St- Pierre, 1998) and acetyl-CoA:4-*O*-deacetylvindoline 4-*O*-acetyltransferase (DAT) (St-Pierre *et al.,* 1998) both restricted to the laticifer and idioblasts cells in leaves (Fig. 2). Because NMT, DAT and D4H are organ-specifically distributed in mature plants and thus, absent in plant cell cultures, it shows the extent in the regulation of the biosynthetic steps leading to vindoline (St-Pierre and De Luca, 1995). With the vindoline pathway fully elucidated (De Luca *et al.,* 2014) it was successfully expressed in yeast (Qu *et al.,* 2015). From the intermediate 4,21-dehydrogeissoschizine, stemmadenine might be formed, which then leads to the formation of catharanthine and vindoline (Meijer *et al.,* 1993a; 1993b). Recently, two enzymes in the branching point at stemmadenine were described to isomerize, deacetoxylate and cyclize stemmadenine into catharanthine and tabersonine via catharanthine synthase (CT) and tabersonine synthase (TS) (Caputi *et al.,* 2018) (Fig. 2). Qu *et al.* (2018) reported the elucidation of the full pathway from 19*E*-geissoschizine to tabersonine and catharanthine. The steps leading to the dimeric TIA vinblastine and vincristine involve i) the dimerization of catharanthine and vindoline to α-3',4'-

anhydrovinblastine catalyzed by the class III peroxidase α-3',4'-anhydrovinblastine synthase (PRX1) that ii) hydroxylates the double bond yielding vinblastine and iii) oxidizes the *N*-methyl group yielding vincristine thus marking the end of TIA biosynthesis in *C. roseus*. Expression of *PRX1* occurs only in petals, reproductive organs and leaves, further supporting that the occurrence of TIA downstream of vindoline only occurs in fully developed tissues (Costa *et al.,* 2008) (Fig. 2).

After some 40 years of research on the biosynthesis of TIA in *C. roseus,* most of the pathways have now been characterized and the identified genes have been overexpressed in plants and yeast. Though overexpression of the genes in other plants and yeast has been successful, in terms of yields the production is not of interest. Only by using precursor feeding to transgenic plants or yeast containing a few biosynthetic genes, commercially interesting levels of an alkaloid have been achieved, *e.g.* strictosidine that is produced in *E. coli* or in yeast (Kutchan *et al.,* 1988). A yeast containing strictosidine synthase was able to produce 2 g/L of this alkaloid in 3 days after feeding with tryptamine and berry juice from *Symphoricarpos albus*, which contains about 2% of secologanin and carbohydrates (Geerlings *et al.,* 2001). Apparently, the TIA pathway involves many steps localized in different cells and cellular compartments making the regulation extremely complex, as it involves transport of intermediates between cellular compartments and cells. Transport is in part driven by diffusion, in part by highly selective transporters of different classes that for each single intermediate have different affinity. Signal molecules like jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) may affect various steps in the biosynthesis, changing carbon fluxes through the different parts of the metabolic network. In this chapter, we will describe the effect on TIA biosynthesis after adding JA to *C. roseus* cell cultures.

4.1.2 Effect of jasmonic acid on TIA biosynthesis in *Catharanthus roseus*

The plant stress hormone JA and its methyl ester (MeJA) have an inducing effect on the signal transduction pathway that induces the biosynthesis of TIA in *C. roseus.* Both jasmonates (JAs) induce several of the known TIA biosynthetic pathway genes (Fig. 2) (Collu *et al.,* 2001; Miettinen *et al.,* 2014; Simkin *et al.,* 2013), which results in transiently higher levels of TIA in cell suspension cultures of *C. roseus* (Lee-Parsons *et al.,* 2004). The coordinated expression of biosynthetic genes is mediated through the ORCA transcriptional factors (Menke *et al.,* 1999a), where only ORCA2 and ORCA3 are induced by JA (Li *et al.,* 2013; Memelink *et al.,* 2001; van der Fits and Memelink, 2000) but not via ORCA1. This transcription factor may have a very different function, which is supported by its homology with the *Arabidopsis* dehydration-responsive elements (DREB2A and DREB2B), which are involved in drought-responses (Liu *et al.,* 1998). ORCA2 transactivates the STR promoter, a jasmonate- and elicitor-responsive promoter element (JERE) after induction with MeJA (Menke *et al.,* 1999a). Overexpression of ORCA3 also induced the biosynthetic genes *STR* and *TDC* and is functionally and structurally similar to ORCA2 (Zhou and Memelink, 2016). ORCA3 controls the transcription of

several genes in both the shikimate and mevalonate pathway such as *AS, DXS, D4H, STR* and *TDC* (van der Fits and Memelink, 2000; Pan *et al*., 2012) and ORCA2 controls the expression of *AS, TDC, LAMT, STR, T16H, D4H* and *PRX1* (Li *et al.,* 2013) (Fig. 1 and 2). Expression of *ORCA3* followed by JA treatment has been confirmed in cells of *C. roseus* (Menke *et al.,* 1999b) with a maximal effect after 8 h of treatment with MeJA (Shukla *et al.,* 2010). Moreover, biotic and abiotic elicitors have also been able to induce the gene expression of *TDC, STR* and *SGD* (Ramani and Jayabaskaran, 2008; Ramani and Chelliah, 2007; Smith *et al.,* 1987b; Zhao *et al.,* 2001a) thus demonstrating the vast effect of JA on plant metabolism as it induces both primary and secondary metabolism at the level of gene expression where ORCA3 acts as the central regulator (Memelink *et al.,* 2001). Cell suspension cultures of *C. roseus* overexpressing ORCA3 significantly accumulated more tryptophan and tryptamine but no TIA were found, only feeding of loganin caused an increase in TIA (van der Fits and Memelink, 2000).

The accumulation of TIA can be considered as a late response in the JAs-mediated stress response in *C. roseus,* where the coordinated expression of *TDC, STR* and *SGD* genes takes place after 2 h of induction (Collu *et al.,* 2001; Wei, 2010) and the accumulation of TIA is detected in significant amounts after 4-24 h (Moreno *et al.,* 1996; Vázquez-Flota *et al.,* 2009). The early steps in the JA-mediated response after wounding include a rapid burst of JAs propagating from locally wounded to distally unwounded leaves that takes place within 30 sec (Glauser *et al.,* 2009), preceding any transcriptional activity. As a part of an integrative study on the fast JA-response, we studied TIA accumulation after induction with JA of cell suspension cultures of *C. roseus* to establish a time line of events in the first 24 hours including both primary (Chapter 3) and secondary metabolism (this chapter).

4.2 EXPERIMENTAL

4.2.1 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 in 40% ethanol (v/v) or 150 µL of 40% ethanol (v/v) (mock), or nothing (untreated) 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 until further analysis.

4.2.2 Chemicals used for cell suspension cultures

The chemicals used for macro 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 BV, Uitgeest, The Netherlands). The chemicals used for micro salts, H_3BO_3, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, Na_2EDTA (Merck; Darmstadt, Germany) and $FeSO_4.7H_2O$ (Brocades-ACF Groothandel NV, Maarssen, The Netherlands), were dissolved into one solution, and KI, $NaMoO_4.2H_2O$, $CuSO_4.5H_2O$ and $CoCl_2.6H_2O$ (Merck; Darmstadt, Germany) were dissolved into another solution to avoid problems of insolubility. Thiamine-di-HCl was from Janssen Chimica (Geel, Belgium), pyridoxine-HCl was from Sigma-Aldrich (St. Louis, MO, USA); 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 (Haarlem, The Netherlands).

4.2.3 Alkaloid standards

Strictosidine and secologanin were provided by Phytoconsult (Leiden, The Netherlands); loganic acid, tabersonine and vindoline were purchased from PhytoLab (Vestenbergsgreuth, Germany); tryptamine was purchased from Sigma-Aldrich Chemical (Steinheim, Milwaukee, WI, USA); tryptophan and ajmalicine were purchased from Sigma-Aldrich (St. Louis, MO, USA); serpentine was purchased from Roth (Karlsruhe, Germany) and catharanthine was a kind gift of Pierre Fabre (Gaillac, France).

4.2.4 Alkaloid and precursor extraction for HPLC

A modified extraction protocol was followed after Moreno *et al.* (1993). Briefly, freeze-dried samples of 50 mg were extracted twice with 5 mL of methanol, vortexed for 1 min, sonicated for 20 min and then centrifuged for 30 min at 3,500 rpm. Pooled samples were reduced to dryness under reduced pressure. To the dried extract 500 µL of 1 M H_3PO_4 was added and the suspension was thoroughly homogenized and then transferred to an Eppendorf tube for centrifugation for 10 min at 13,000 rpm. Extracts were filtered with a 0.2 µm PTFE membrane and then 50 µL were analyzed by HPLC.

4.2.5 HPLC analysis of TIA

Chromatographic separations were carried out on a 250 mm x 4.60 mm, 5 µm Gemini-NX C18 (Phenomenex Inc., Torrance, California, USA) column and a guard column filled with RSil C18 HL (Uetikon) at room temperature. Chromatographic methods were adapted from Tikhomiroff and Jolicoeur (2002). Elutions were performed at a flow rate of 1.5 mL/min. Solvents used for TIA (strictosidine, ajmalicine, serpentine, catharanthine, tabersonine, vindoline, vincristine and vinblastine) analysis were 5 mM Na_2HPO_4 in water adjusted to pH 6 with H_3PO_4; (Solvent A) and acetonitrile (v/v) (Solvent B). Elution was as follows: from 0-20 min, linear gradient from 80:20 to 20:80 (v/v) (A:B); 20-25 min, isocratic elution with 20:80 (v/v) (A:B); 25-30 min, linear gradient from 20:80 to 80:20 (v/v) (A:B); 30-31 min, isocratic elution at 80:20 (v/v) (A:B). Precursors (tryptamine, tryptophan,

loganic acid, loganin and secologanin) were analyzed using an isocratic elution of 85:15 (v/v) of 0.01 M H_3PO_4 in water (Solvent A) and acetonitrile (Solvent B). Elutions were performed at a flow rate of 1.5 mL/min and the injection volume was 50 µL for both methods. The HPLC system was an Agilent 1200 Series consisting of a G1310A binary pump, a G1329A autosampler, a G1322A degasser and a G1315D photo-diode array detector (DAD) controlled by ChemStation software (Agilent v. 03.02; all from Agilent Technologies Inc., Santa Clara, CA, USA). Ultraviolet (UV)-spectra of all peaks were collected (220-320 nm) and chromatograms were recorded at 220, 254, 280, 306 and 320 nm. Detection of target peaks was achieved by comparison of their absorbance with that of reference standards. Every sample was injected once.

4.2.6 Extraction procedures for UHPLC-TOF-HRMS analysis

Approximately 10 mg of lyophilized plant material was extracted twice with 2 mL of isopropanol, vortexed for 1 min and 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 (Waters Sep-Pak C18, 1 mL, 100 mg; Milford, MA, USA) purification, 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 used for UHPLC-TOF-HRMS analyses.

4.2.7 Quantification of TIA

External calibration curves of strictosidine, ajmalicine-HCl, serpentine-HCl, tabersonine-HCl, catharanthine sulphate and loganic acid were constructed over different concentration levels for each TIA and their UV spectra were recorded at 280, 306 and 320 nm. Working solutions were prepared on 0.01 M H_3PO_4 and each sample was analyzed once *i.e.* there are no technical replicates. Peaks were identified based on chromatographic retention times and spectral data compared to their respective reference standards. Detection and quantification were based on peak area of each TIA.

4.2.8 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, St Louis, MO, USA) 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 (Milford, MA, USA) 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. Every sample was injected once.

4.2.9 Data handling and analysis

Raw LC–MS data were processed using MarkerLynx® software (Waters, Milford, MA, USA). Before statistical analysis, distributions were tested for normality using the Shapiro-Wilk test ($p < 0.05$). A one-way ANOVA with Tukey's multiple comparison test was applied to test: (a) significant differences in contents of TIA between treatments in JA-treated, mock-treated and untreated cells in each time point and, (b) differences of TIA in JA-treated cells compared against to those of JA-treated cells at all time points. All statistical tests were performed in GraphPad Prism software (v. 8.4.3.686, La Jolla, CA, USA). Differences with $p < 0.1$ and $p < 0.05$ were considered statistically significant and are indicated as * and **, respectively. ANOVA results are shown in Table 4.7.1. (a) and Table 4.7.2. (b) in the supplemental information section.

4.3 RESULTS AND DISCUSSION

4.3.1 α-Methylene-indoline alkaloids in cell suspension cultures of *C. roseus*

Alcoholic extractions allowed the detection and quantification of 10 different TIA and 2 precursors by means of their UV spectra and high-resolution (HR) MS data (Table 1 and supplemental Figure 4.7.3.). Seven peaks were noticed and distinguished only by their different retention times (α-TIA-1-7). According to their absorbance under UV light, three maxima were observed at 225, 300 and 325 nm and a deep minimum at 250 mAU (Saxton, 1965; Hisiger and Jolicoeur, 2007) (Fig. 3), which is characteristic of alkaloids possessing an α-methylene indoline chromophore, constituted by a double bond conjugated with a carbonyl group in the α position of the indoline nitrogen atom (Fig. 4A). Moreover, these alkaloids are also present in their *N*-oxide form and thus have a similar UV spectrum (Malikov and Yunusov, 1977; Schripsema *et al.,* 1987). Some examples of these alkaloids are shown in Fig. 4B-E. Several of these, like tubotaiwine, lochnericine (van der Heijden *et al.,* 2004; 1989; Giddings *et al.,* 2011), condylocarpine (El-Sayed *et al.,* 2004) and akuammicine (Scott *et al.,* 1980; Stöckigt and Soll, 1980) have been reported to accumulate in cell suspension and tissue cultures of *C. roseus* (van der Heijden *et al.,* 1989). Among these, based on the exact mass of *m/z* 353.185969 $[M+H]^+$ derived from the molecular formula $C_{21}H_{24}N_2O_3$, the possible α-TIA candidates are 19-hydroxytabersonine, akuammigine and lochnericine, all found in cell suspension and tissue cultures of *C. roseus* (van der Heijden *et al.,* 1989; 2004). However, the aim of this work was not to identify each trace alkaloid present in our cell system but to get a more general overview of the main TIA

Table 1. Detection parameters of TIA in cell suspension cultures of *Catharanthus roseus* by HPLC-DAD and UHPLC-TOF-HRMS

TIA	Wavelength (nm)	HPLC-DAD RT (min)	UHPLC-TOF-MS RT (min)	Dynamic range (µmol/g DW)	$[M+H]^+$	Molecular formula
Strictosidine	225, 275	4.83	1.55	2.91-20.89	530.2264	$C_{27}H_{34}N_2O_9$
Loganic acid	225	5.24	0.78	0.35-3.35	375.1277	$C_{16}H_{24}O_{10}$
Serpentine	250, 306	6.78	1.61	1.07-2.03	349.1549	$C_{21}H_{20}N_2O_3$
α-TIA-1	225, 300, 325	11.43	1.14	0.14-1.32	353.1863	$C_{21}H_{24}N_2O_3$
α-TIA-2	225, 300, 325	11.94	1.27	0.08-0.20	353.1863	$C_{21}H_{24}N_2O_3$
Catharanthine	225, 280	13.27	1.60	0.01-0.20	337.1916	$C_{21}H_{24}N_2O_2$
α-TIA-3	225, 300, 325	17.15	1.36	0.78-1.56	353.1862	$C_{21}H_{24}N_2O_3$
α-TIA-4	225, 300, 325	19.95	1.41	0.009-0.39	353.1862	$C_{21}H_{24}N_2O_3$
Tabersonine	225, 300, 325	21.24	1.66	0.16-0.78	337.1914	$C_{21}H_{24}N_2O_2$
α-TIA-5	225, 300, 325	22	1.55	0.009-0.01	353.1861	$C_{21}H_{24}N_2O_3$
α-TIA-6	225, 300, 325	23	1.62	0.01	353.1877	$C_{21}H_{24}N_2O_3$
α-TIA-7	225, 300, 325	24.65	1.65	0.001-0.03	353.1864	$C_{21}H_{24}N_2O_3$

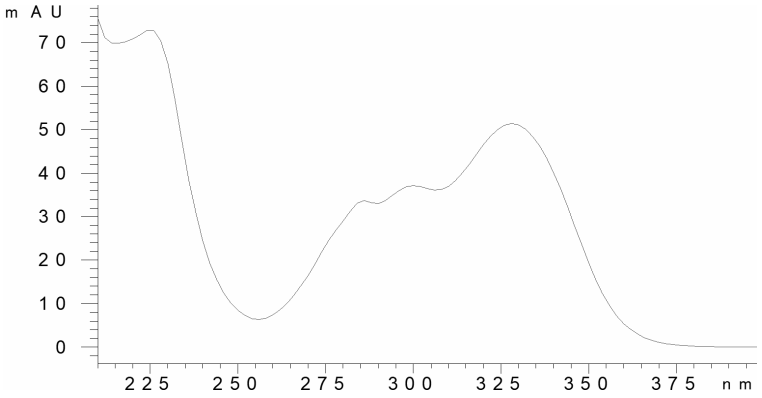

Figure 3. UV spectra of a α-TIA (1-7) detected in cell suspension cultures of *Catharanthus roseus* analyzed by HPLC-DAD.

accumulated after JA treatment. Further studies including their isolation and structural elucidation by NMR- and MS-approaches are needed to fully characterize them.

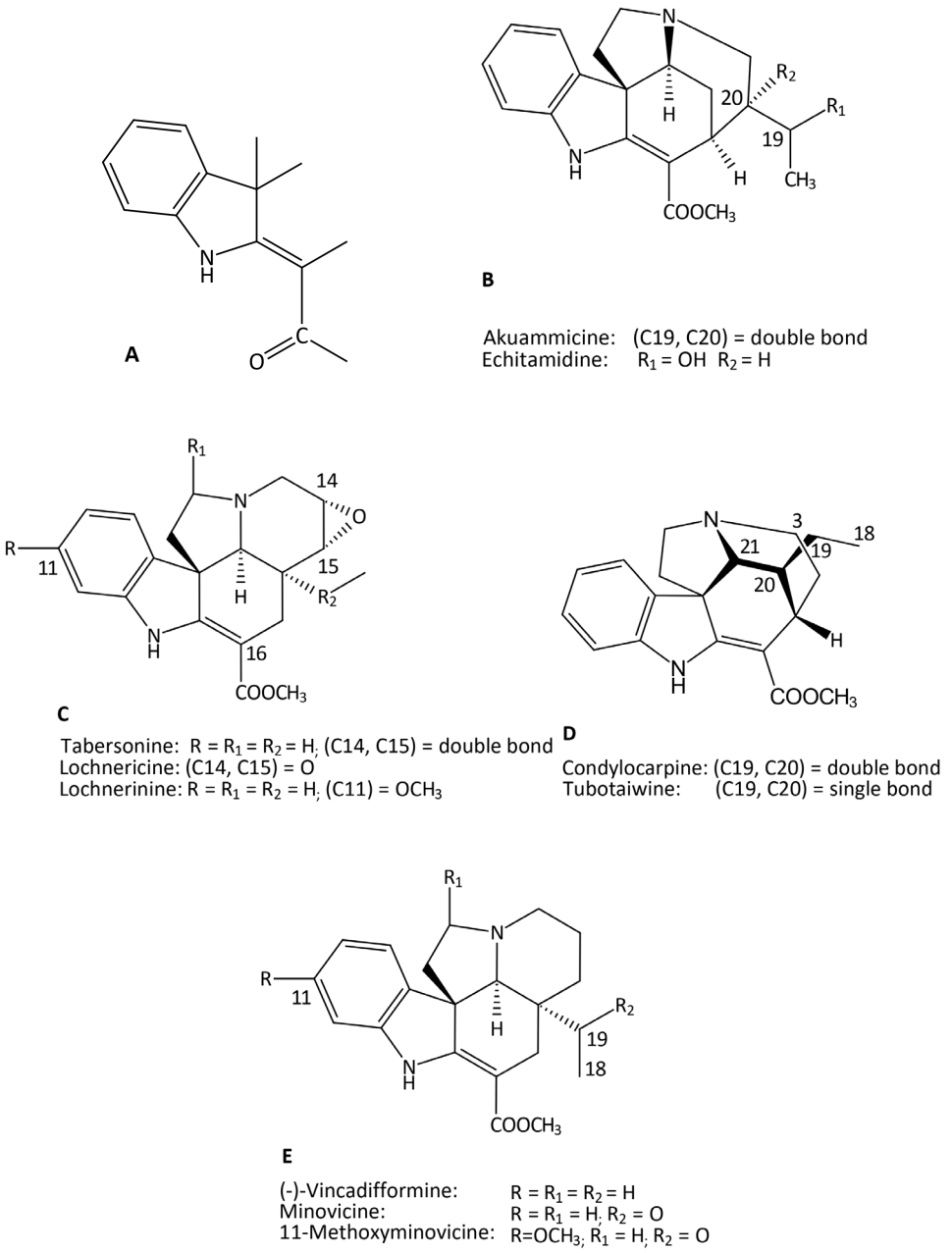

Figure 4. Terpenoid indole alkaloids with a α-methylene indoline chromophore possibly accumulated in cell suspension cultures of *Catharanthus roseus.* **A.** Typical indoline system. **B**. Alkaloids with a C20 skeleton. **C**. Alkaloids with a C21 skeleton (Tabersonine and Lochnericine) and C22 skeleton (Lochnerinine). **D**. Alkaloids with a C20 skeleton. **E**. Alkaloids with a C21 skeleton.

4.3.2 Effect of jasmonic acid on the accumulation of TIA in *C. roseus*

Jasmonic acid treatment was applied to cell suspension cultures of *C. roseus* on the 4th day after subculture. Strictosidine was by far the most abundant TIA in all treatments and controls (1 or 2 orders of magnitude higher than the other TIA found in these experiments) with the highest levels of 20.89 µmol/g DW (at 360 min) and 20.87 µmol/g DW (at 90 min), both in JA-treated cells and followed by mock-treated cells at 360 min (20.69 µmol/g DW) (Fig. 5). Levels of strictosidine in JA-treated cells followed, more or less, the trend of untreated and mock-treated cells at 1440 min. There was a 2.2-fold decrease in strictosidine levels at 1440 min in JA-treated cells if compared to JA-treated cells at t=0 (Fig. 5), although significant differences were observed in JA-treated cells at 1440 min compared to JA-treated cells at 5, 90 and 360 min. At 30 min in JA-treated cells, levels of strictosidine showed a 0.7-fold decrease when compared to mock-treated cells and a 0.9-fold decrease in comparison to untreated cells at 30 min. Levels of strictosidine in JA-treated cells at 90 and 60 min, were significantly higher than JA-treated cells at 30 min. Loganic acid showed lower levels at 30 and 90 min in JA-treated cells in comparison to untreated cells and the mock treatment at these time points. Moreover, levels at 30 min in JA-treated cells were significantly lower than at t=0 and 5 min in JA-treated cells. When compared to JA-treated cells at t=0, a 2.9-fold difference was observed in JA-treated cells at 90 min with a decreasing and significative trend towards the end of the experiment if compared to JA-treated cells at t=0. Neither tryptophan nor tryptamine were detected in this cell line in complete agreement with results of Saiman et al. (2014; 2015). Contents of tabersonine and α-TIA-1 showed similar trends in the JA and mock treatments and in untreated cells over time. JA-treated cells showed for both TIA an increase towards the end of the experiment showing a 1.8-fold increase compared to the mock and a 2-fold increase compared to untreated cells, both at 1440 min (Fig. 5). Levels of α-TIA-2 and α-TIA-3 showed no clear trend, though at 1440 min JA-treated and mock-treated cells did not contain any measurable amount of α-TIA-2. Though accumulation of serpentine seemed to be suppressed in the JA-treated cells (0-360 min), at the end of the experiment it was again about at the same level as untreated and mock-treated cells. Overall, there was a 1.6-fold increase in contents of serpentine in JA-treated cells at 1440 min in comparison to JA-treated cells from the previous time point of observation, and a significant 1.7-fold increase when compared to JA-treated cells to all previous time points. A 0.8-fold decrease in levels of serpentine was seen after 30 min in both JA-treated cells and mock-treated cells in comparison to the control at 30 min. After 90 min, levels of serpentine were lower than untreated and mock-treated cells. However, in the case of catharanthine, higher levels were visible after 1440 min in JA-treated cells when compared to the control and to JA-treated cells at t=0, 90 and 360 min. After 90 and 360 min, levels of catharanthine were significantly lower (more than 10-fold) for untreated, mock-treated and JA-treated cells when compared to earlier time points. There is no clear explanation for this, as other TIA, for as far analyzed, did not show any major changes at these time points. Traces of α-TIA-4-7 were noticed in most treatments though showing a high variation amongst biological replicates. Furthermore, because of their unconfirmed identity and low levels, their quantification was

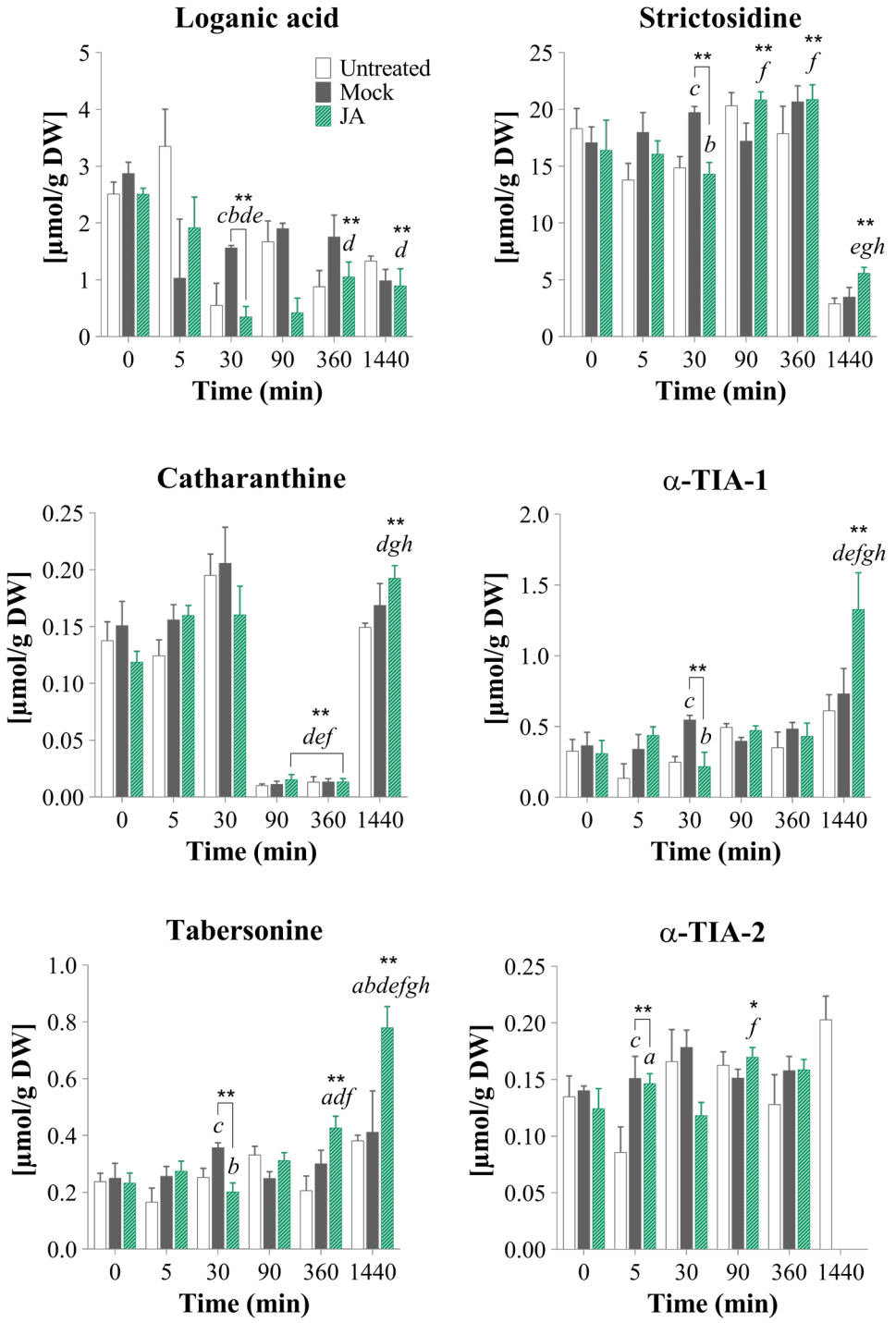

Figure 5. Continued

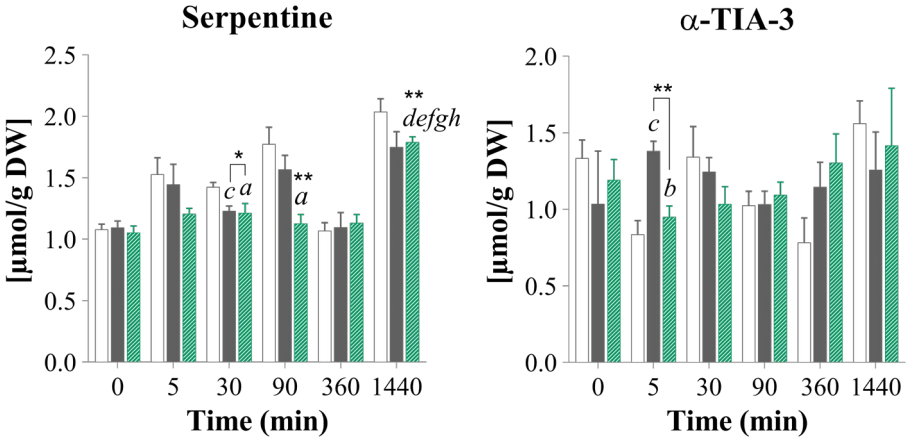

Figure 5. HPLC-DAD analysis of terpenoid indole alkaloids in cell suspension cultures of *Catharanthus roseus* treated with jasmonic acid. Values are the mean ± standard error of mean (SEM) of four biological replicates each analyzed once. Significant results are marked with superscripts (one-way ANOVA with Tukey's post hoc test, $*p < 0.1$, $**p < 0.05$).
[a] JA-treated significantly different from untreated at the same time point of observation.
[b] JA-treated significantly different from mock at the same time point of observation.
[c] Mock significantly different from untreated at the same time point of observation.
[d] JA-treated significantly different from JA-treated at t=0.
[e] JA-treated significantly different from JA-treated at t=5.
[f] JA-treated significantly different from JA-treated at t=30.
[g] JA-treated significantly different from JA-treated at t=90.
[h] JA-treated significantly different from JA-treated at t=360.
[i] JA-treated significantly different from JA-treated at t=1440.
α-TIA 2 in mock and JA treatments at 1440 min was below the detection limit.
Lower levels in catharanthine at 90 and 360 min is due to the absence of this alkaloid in some of the replicates in all treatments.

not possible, although their levels are shown in Figure 6 as areas under the curve (AUC) analyzed by UHPLC-TOF-HRMS. α-TIA-4 increased over time with significant differences in JA-treated cells after 360 min in comparison to JA-treated cells at 5 min. α-TIA-5 showed an increase towards the end of the experiment with significantly higher contents after 360 min when compared to untreated cells at the same time point and to JA-treated cells at 5 and 30 min. A similar trend was observed for α-TIA-6 and α-TIA-7, with peaking levels after 360 min in JA-treated cells. From these four α-TIA, only α-TIA-6 had the lowest levels whereas α-TIA-5 had the highest levels in JA-treated cells at 360 min. Interestingly, these α-TIA showed their highest levels at 360 min in JA-treated cells and none of them seemed to be affected by the mock treatment.

In previous experiments with the same cell suspension cultures (CRPP) of *C. roseus* supplemented with either glucose or sucrose, levels of loganic acid, strictosidine, catharanthine, tabersonine and serpentine were found to be higher in sucrose-supplemented growth medium (Saiman *et al.,* 2014). However, in JA-treated studies in the same cell line but fed with glucose, only loganic acid, strictosidine, serpentine and tabersonine were reported. These precursors and TIA showed

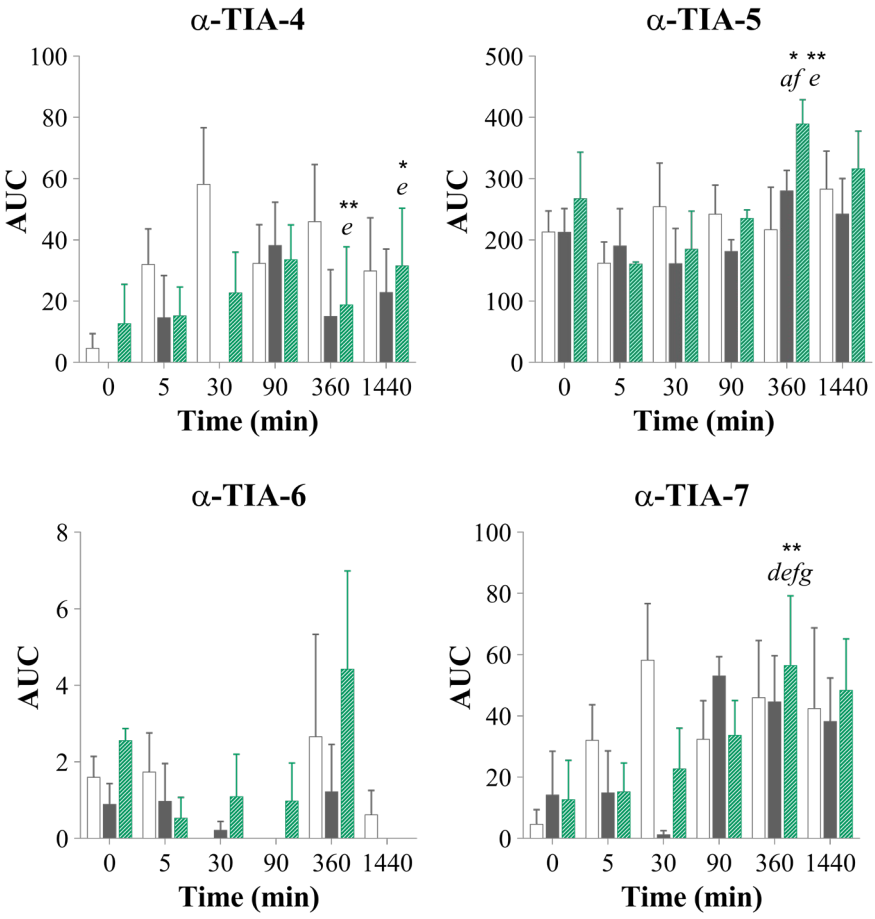

Figure 6. UHPLC-TOF-MS analysis of terpenoid indole alkaloids in cell suspension cultures of *Catharanthus roseus* treated with jasmonic acid. Values are the mean ± standard error of mean (SEM) of four biological replicates each analyzed once. Significant results are marked with superscripts (one-way ANOVA with Tukey's post hoc test, $*p < 0.1$, $**p < 0.05$). α-TIA-6 was not tested due to insufficient data for ANOVA calculations.
[a] JA-treated significantly different from untreated at the same time point of observation.
[b] JA-treated significantly different from mock at the same time point of observation.
[c] Mock significantly different from untreated at the same time point of observation.
[d] JA-treated significantly different from JA-treated at t=0.
[e] JA-treated significantly different from JA-treated at t=5.
[f] JA-treated significantly different from JA-treated at t=30.
[g] JA-treated significantly different from JA-treated at t=90.
[h] JA-treated significantly different from JA-treated at t=360.
[i] JA-treated significantly different from JA-treated at t=1440.

increased levels after 72 h in the JA-treated cells when compared to the untreated (Saiman *et al.,* 2015). Furthermore, experiments with the cell line A12A2 of *C. roseus* showed increased levels of strictosidine and ajmalicine after elicitation with MeJA (El-Sayed and Verpoorte, 2002). Further investigations on

cell suspensions of *C. roseus* treated with 100 µM MeJA showed a 300% increase in serpentine (Lee-Parsons *et al.,* 2004).

According to our previous work and those reported in the literature, 100 µM JA or MeJA is the optimum concentration to elicit secondary metabolism when added in a particular frame of time during cell growth, usually after 4 to 6 days of subculture, *i.e.* in the exponential growth phase when cells are rapidly dividing and growing(Lee-Parsons *et al.,* 2004; Gundlach *et al.,* 1992). Other studies have reported an increased TDC activity after elicitation with 10 µM MeJA or 5 ppm of ET in suspension cultures of *C. roseus,* leading to an increased accumulation of ajmalicine but not of catharanthine within the first 24 h and returning to initial values after 48 h. Even though both treatments lead to the accumulation of TIA, it is noteworthy to observe a differential response in TDC activity with both elicitors. After ethylene exposure, the activity increased transiently after 12 h and peaked at 24 h, whereas exposure to MeJA showed the highest TDC activity after 12 h (Vázquez-Flota *et al.,* 2009). Ajmalicine production is highly inducible by MeJA in cell suspension cultures of *C. roseus* where the highest yield (4.75 mg/L) was observed when cells were treated with 3 mM of $CaCl_2$ and 100 µM of MeJA (Lee-Parsons and Ertürk, 2005). Higher concentrations of this ion showed a negative effect on the accumulation of this alkaloid, which might be explained by the fact that the cytosolic pools of Ca^{2+} may alter signaling processes in which they act as a second messenger, along with protein phosphorylation, in hormone and stress signaling (Arimura and Maffei, 2010; Rudd and Franklin-Tong, 2001; Sun *et al.,* 2010). Another major role of Ca^{2+} in alkaloid biosynthesis induction in *C. roseus* after fungal elicitation is to induce mRNA accumulation of the transcriptional factors *Catharanthus roseus* Box P Binding Factor-like protein 1 (CrBPF-1) and ORCAs that regulate the expression of *STR* in a JA-independent signal transduction pathway (van der Fits *et al.,* 2000).

Besides the effect of JA on TIA accumulation, mock treatment (40% v/v ethanol) transiently induced levels of strictosidine, tabersonine, α-TIA-1, α-TIA-2 and α-TIA-3 when compared to those of JA at the same time point of observation. Most changes were significant due to the treatment and were noticed between 5 and 90 min (Fig. 5) as it was also observed in the fatty acid (FA) profiles of cells (Chapter 3 of this thesis). Both JA- and mock-treatment (40 % v/v ethanol) of the cells induced changes. Similarly, Saiman et al. (2015) found that feeding cell suspension cultures of *C. roseus* with 20% (v/v) of ethanol, increased levels of loganic acid, strictosidine, serpentine and tabersonine at 6 and 72 h compared to the time of treatment. Yet, the addition of JA resulted in an equal or stronger increase compared to the mock, with significantly higher levels for strictosidine, serpentine and tabersonine at 72 h. Furthermore, Saiman (2014) observed a stronger induction of TIA after 72 h of feeding cell suspensions of *C. roseus* with a solution of 50 % (v/v) of ethanol and JA dissolved in 50 % (v/v) of ethanol. Levels of strictosidine, serpentine, catharanthine and tabersonine increased to similar levels as those in JA-treated cells after 72 h. Moreover, levels of these TIA in mock-treated cells were two times higher than those after 24 h of treatment.

Elicitation of suspension cultures of *C. roseus* with mycelial homogenates *i.e. Phytium aphanidermatum, Alternaria zinnae, Verticillium dahlie, Rhodotor ularubra, Eurotium rubrum* to name a few, are able to induce the accumulation of strictosidine lactam, ajmalicine, lochnericine, catharanthine and tabersonine, which includes a rapid and transient increase in the activities of *TDC* and *STR* (Shukla *et al.,* 2010; Eilert *et al.,* 1986; 1987; Tallevi *et al.,* 1986; DiCosmo *et al.,* 1987; Zhao *et al.,* 2001b). Interestingly, the induction of *STR* activity preceded that of *TDC* by 60 h suggesting that endogenous levels of tryptamine might regulate the expression of *TDC* (Eilert *et al.,* 1987; Moreno *et al.,* 1996). All these observations are supported by the fact that fungal elicitors are able to induce endogenous levels of JA in suspension cultures of *C. roseus* (Mueller *et al.*, 1993) which is enough to induce the expression of the *STR* gene (Menke *et al.,* 1999b).

In a large-scale transcriptomic and metabolomic profiling analysis of MeJA-elicited cells of *C. roseus* a gene-to-metabolite network confirmed previous knowledge concerning the coordinated regulation of *TDC* and *STR* by MeJA up to 16-hydroxytabersonine (Rischer *et al.,* 2006). Later steps *e.g. D4H* and *DAT* expression, are not induced by MeJA in cell suspension cultures of *C. roseus* (Vázquez-Flota *et al.,* 2002) explaining the absence of vindoline. Nonetheless, contents of 16-hydroxytabersonine, 16-methoxytabersonine, 16-methoxy-2,3-dihydro-3-hydroxytabersonine and desacetoxyvindoline were significantly induced in *C. roseus* leaves after MeJA treatment (Zhang *et al.,* 2018b*).* It thus seems that the JA effect differs with the type of cell. Rischer *et al.* (2006) reported other metabolic pathways affected by MeJA elicitation. They classed these pathways and reported the following distribution: cell organization and defense (2.4%), metabolism and energy (28%), protein synthesis (3.1%), signal transduction (3.1%), transport (3.6%) and transcription (4.8%).

4.4 CONCLUSIONS

These results extend the knowledge on TIA accumulation in cells of *C. roseus* after JA-induction within the first 24 h. Considering the iridoid precursor loganic acid, results showed a decreasing trend towards 1440 min in all treatments and untreated cells. Strictosidine, as early intermediate, was by far the major TIA with levels up to 20.87-20.89 µmol/g DW at 90 and 360 min in JA-treated cells and almost equally high levels in untreated and mock-treated cells. However, at 1440 min, levels dropped to 2.91, 3.5 and 5.6 µmol/g DW in untreated-, mock- and JA-treated cells, respectively. Significantly increased levels of tabersonine were observed towards the latest time points (360-1440 min), confirming previous observations made after JA elicitation with the same cell line. Additional increases were observed for serpentine (1.5-fold increase), tabersonine (1.8-fold increase), catharanthine (10.5-fold increase) and α-TIA-1 (3-fold increase) in JA-treated cells at 1440 min in comparison to levels at 360 min in JA-treated cells; however, these increases do not add up to the decrease in levels of strictosidine, in this time frame. Apparently, changes are limited, and the *de novo* biosynthesis of TIA does not seem to play a major role in the 1440 min time span. Furthermore, the effect of mock on TIA contents was only

visible between the 5-30 min time points, thus showing that all changes in TIA profiles towards the end of the experiment were the result of treatment with JA. Based on these observations, we support the idea that two independent time-events occur after JA induction. In the early response *i.e.* after 5-30 min of induction, no major effects are observed for TIA levels; only the precursor loganic acid seems to be decreased and the second event, taking place after 1440 min with increased levels of serpentine, tabersonine, catharanthine and α-TIA-1.

4.5 ACKNOWLEDGMENTS

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4.7 SUPPLEMENTAL INFORMATION

Table 4.7.1. ANOVA results for each test performed for each terpenoid indole alkaloid in untreated, mock-treated and JA-treated cells per time point of suspension cultures of *Catharanthus roseus*

			Time (min)			
TIA	**0**	**5**	**30**	**90**	**360**	**1440**
Loganic acid	$F_{(2,9)} = 1.487$ $p=0.2767$	$F_{(2,6)} = 2.157$ $p=0.1969$	$F_{(2,9)} = 6.943$ $p=0.015$	$F_{(2,7)} = 3.077$ $p=0.11$	$F_{(2,9)} = 2.225$ $p=0.164$	$F_{(2,9)} = 1.223$ $p=0.339$
Strictosidine	$F_{(2,9)} = 1.185$ $p=0.3491$	$F_{(2,9)} = 2.067$ $p=0.1825$	$F_{(2,9)} = 12.12$ $p=0.0028$	$F_{(2,9)} = 2.781$ $p=0.1147$	$F_{(2,9)} = 0.9161$ $p=0.4344$	$F_{(2,9)} = 1.656$ $p=0.2440$
Catharanthine	$F_{(2,9)} = 0.9815$ $p=0.4115$	$F_{(2,9)} = 2.61$ $p=0.1277$	$F_{(2,9)} = 0.8652$ $p=0.4532$	$F_{(2,9)} = 0.8978$ $p=0.441$	$F_{(2,9)} = 0.0007463$ $p=0.9993$	$F_{(2,9)} = 2.84$ $p=0.1106$
α-TIA 1	$F_{(2,9)} = 0.1019$ $p=0.9041$	$F_{(2,9)} = 2.979$ $p=0.1017$	$F_{(2,9)} = 7.946$ $p=0.0103$	$F_{(2,9)} = 3.462$ $p=0.0767$	$F_{(2,9)} = 0.6027$ $p=0.568$	$F_{(2,9)} = 4.002$ $p=0.0571$
Tabersonine	$F_{(2,9)} = 0.0524$ $p=0.9492$	$F_{(2,9)} = 2.16$ $p=0.1714$	$F_{(2,9)} = 8.734$ $p=0.0078$	$F_{(2,9)} = 2.588$ $p=0.1294$	$F_{(2,9)} = 5.699$ $p=0.0252$	$F_{(2,9)} = 5.511$ $p=0.0274$
α-TIA 2	$F_{(2,9)} = 0.3054$ $p=0.7441$	$F_{(2,9)} = 4.222$ $p=0.0509$	$F_{(2,9)} = 2.66$ $p=0.1237$	$F_{(2,9)} = 0.9877$ $p=0.4095$	$F_{(2,9)} = 1.005$ $p=0.4038$	-
Serpentine	$F_{(2,9)} = 0.1839$ $p=0.8351$	$F_{(2,9)} = 1.799$ $p=0.2201$	$F_{(2,9)} = 4.762$ $p=0.0388$	$F_{(2,9)} = 8.76$ $p=0.0077$	$F_{(2,9)} = 0.1306$ $p=0.8792$	$F_{(2,9)} = 2.581$ $P=0.1301$
α-TIA 3	$F_{(2,9)} = 0.4451$ $p=0.6541$	$F_{(2,9)} = 14.56$ $p=0.0015$	$F_{(2,9)} = 1.239$ $p=0.3347$	$F_{(2,9)} = 0.1902$ $p=0.8301$	$F_{(2,9)} = 2.463$ $p=0.1403$	$F_{(2,9)} = 0.3123$ $p=0.7393$
α-TIA 4	-	$F_{(2,4)} = 0.3316$ $p=0.7358$	-	$F_{(2,6)} = 0.3404$ $p=0.7244$	$F_{(2,2)} = 0.1253$ $p=0.8887$	$F_{(2,3)} = 1.037$ $p=0.4547$
α-TIA 5	$F_{(2,5)} = 0.4015$ $P=0.6821$	$F_{(2,9)} = 0.1732$ $p=0.8437$	$F_{(2,9)} = 0.5789$ $p=0.5801$	$F_{(2,9)} = 1.221$ $p=0.3394$	$F_{(2,9)} = 3.109$ $p=0.0941$	$F_{(2,9)} = 0.3794$ $p=0.6947$
α-TIA 6	$F_{(2,5)} = 2.013$ $p=0.2285$	-	-	-	-	-
α-TIA 7	-	$F_{(2,4)} = 0.3285$ $p=0.7377$	$F_{(2,4)} = 1.111$ $p=0.4134$	$F_{(2,7)} = 0.7344$ $p=0.5134$	$F_{(2,5)} = 1.902$ $p=0.2431$	$F_{(2,5)} = 2.027$ $p=0.2267$

Not tested due to insufficient data for ANOVA calculations: (-).

Table 4.7.2. ANOVA results for tests performed for each terpenoid indole alkaloid against all time points in JA-treated cells of suspension cultures of *Catharanthus roseus*

TIA	**F**	***p***
Loganic acid	$F_{(5,16)} = 6.728$	0.0015
Strictosidine	$F_{(5,17)} = 7.1$	0.0009
Catharanthine	$F_{(5,18)} = 38.96$	<0.0001
α-TIA 1	$F_{(5,18)} = 9.901$	0.0001
Tabersonine	$F_{(5,18)} = 24.82$	<0.0001
α-TIA 2	$F_{(4,15)} = 3.638$	0.029
Serpentine	$F_{(5,18)} = 19.3$	<0.0001
α-TIA 3	$F_{(4,15)} = 0.8712$	0.5038
α-TIA 4	$F_{(5,5)} = 5.631$	0.0405
α-TIA 5	$F_{(5,17)} = 3.381$	0.0266
α-TIA 6	-	-
α-TIA 7	$F_{(5,7)} = 8.065$	0.008

Not tested due to insufficient data for ANOVA calculations: (-).

Figure 4.7.3. UHPLC elution profile of α-TIA-1-7 (top panel) and mass spectrum showing *m/z* 353.1867 (bottom panel) found in cells of suspension cultures of *Catharanthus roseus* analyzed by UHPLC-TOF-MS. Retention times for each α-TIA is as follows: 1.14 min α-TIA-1, 1.27 min α-TIA-2, 1.36 min α-TIA-3, 1.41 min α-TIA-4, 1.55 min α-TIA-5, 1.62 min α-TIA-6, 1.65 min α-TIA-7.