Retrobiosynthetic study of salicylic acid in *Catharanthus roseus* cell suspension cultures

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ISBN 978-90-9021786-4

Printed by PrintPartners Ipskamp B. V., Amsterdam, The Netherlands

Cover: *Catharanthus roseus* (artist: Kathleen O'Ryan; source: Australian National Botanic Gardens).

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PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. P. F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 23 mei 2007
klokke 16.15 uur

door

Natali Rianika Mustafa

geboren te Palembang (Indonesia)
in 1964

Promotiecommissie

Promotor	Prof. dr. R. Verpoorte
Referent	Prof. dr. J-P. Métraux (Université de Fribourg, Switzerland)
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Abbreviations

AcCN	acetonitrile
ADP	adenosine diphosphate
AQ	anthraquinones
AS	anthranilate synthase
ATP	adenosine triphosphate
BA	benzoic acid
C4H	cinnamate 4-hydroxylase
CC	column chromatography
CM	chorismate mutase
COSY	correlated spectroscopy
CPL	chorismate pyruvate-lyase
2,4-D	2,4-dichlorophenoxyacetic acid
DAD = PDA	photodiode array detector
2,3-DHBA	2,3-dihydroxybenzoic acid
2,3-DHBAG	2,3-dihydroxybenzoic acid glucoside
DMAPP	dimethylallyl diphosphate
DW	dry weight
DXR	1-deoxy-D-xylulose 5-phosphate reductoisomerase
DXS	1-deoxy-D-xylulose 5-phosphate synthase
E4P	erythrose-4-phosphate
EMP	Embden-Meyerhof-Parnas
EtOAc	ethylacetate
ESI	electrospray ionization
FAB	fast atom bombardment
FW	fresh weight
GA	gallic acid
GABA	γ-amino butyric acid
GC	gas chromatography
G10H	geraniol 10-hydroxylase
HBA	hydroxybenzoic acid
HMBC	heteronuclear multiple bond correlation
HMGR	3-hydroxy-3-methylglutaryl-CoA reductase
HMQC	heteronuclear multiple-quantum coherence
HR	hypersensitive reaction
IAA	indole-3-acetic acid
ICS	isochorismate synthase

IEC	ion exchange chromatography
IPL	isochorismate pyruvate-lyase
IPP	isopentenyl diphosphate
ISR	induced systemic resistance
JA	jasmonate
MECS	2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase
MeJA	methyl jasmonate
MeOH	methanol
MEP	methyl-erythritol phosphate
MS	mass spectrometry
M&S	Murashige & Skoog
NAA	1-naphtaleneacetic acid
NMR	nuclear magnetic resonance
OMT	*O*-methyltransferase
ORCA	octadecanoid responsive *Catharanthus* AP2-domain
PAL	phenylalanine ammonia-lyase
PC	paper chromatography
PCA	principal component analysis
PEP	phosphoenolpyruvate
3PGAL	glyceraldehyde-3-phosphate
PP-ED	pentose phosphate-Entner-Doudoroff
PR protein	pathogenesis related protein
RP-HPLC	reversed phase high performance liquid chromatography
RT-PCR	reversed transcription-polymerase chain reaction
SA	salicylic acid
SAG	salicylic acid glucoside
SAR	systemic acquired resistance
SH	Schenk and Hildebrandt
STR	strictosidine synthase
TDC	tryptophan decarboxylase
TIA	terpenoid indole alkaloid
TLC	thin layer chromatography
TSP	trimethylsilyl propionic acid Na salt
UV	ultra violet

Chapter 1

General Introduction

A plant is a living organism able to convert inorganic material into organic molecules necessary for the life of plant itself and serves as food for e.g. insects, animals and humans. Plants also provide medicines, food additives, flavors, fragrances, pigments, insecticides, paper, fibers, rubber and many other commodities. However, our knowledge about plants with their enormous diversity is still limited in many aspects. So, still many novel products might be obtained from plants, however, this is hampered by the rapid loss of plant diversity on earth due to e.g. deforestation. The sustainable exploitation of plants for food and medicines requires extensive knowledge about plants. As the world's population grows to an estimated 9 billions people in 2050 (Cordell, 2002), the availability of food and medicines for all people in the future should be a concern to all of us. Up to now, plants remain a primary source of medicines for most people in the world (Cordell, 2002). Therefore, research in plant science is of great importance for human health, both for the production of healthier food and for development and production of medicines.

The secondary metabolites that are the source for e.g. pharmaceuticals, food additives or flavors, are species-specific and play a role in the interaction of a plant with its environment (Verpoorte, 1998). Examples are compounds aimed at attraction of pollinators (e.g. insects) or to defend against invaders (e.g. pathogens). An example of the role of secondary metabolism, which is also the basis of the present study, is the plant defense against infections with viruses or microorganisms. The production of secondary metabolites for plant defense such as phytoalexins can be a result of so-called systemic acquired resistance (SAR), an inducible broad-resistance to pathogens. SAR is activated after the formation of a necrotic lesion in leaves as part of the hypersensitive response (HR) to an infection. SAR is associated with the

expression of SAR genes responsible for SAR proteins; many belong to the class of pathogenesis-related (PR) proteins e.g. acidic PR1 proteins, which have antimicrobial activity (reviewed by Ryals *et al*., 1996). Besides the expression of SAR genes as a marker of SAR, SAR is also known to employ salicylic acid (SA) as a signal compound. Another inducible systemic resistance employing other signal compounds like e.g. jasmonate (JA) and ethylene (ET) is called induced-systemic resistance (ISR), which is known to activate genes encoding e.g. proteinase inhibitors and defensins (van Wees *et al*., 2000). Nitric oxide (NO), a signal compound for immune responses in animals, was shown also to mediate plant defense responses against pathogens (Durner and Klessig, 1999). Interaction between signal compounds can occur in a plant generating a systemic resistance as it was reviewed by e.g. Pieterse *et al*. (2001) and Kunkel and Brooks (2002). For example, SA and JA can activate the same genes in *Arabidopsis*. Several genomic studies showed that both SA signaling- and JA signaling pathways need activation of the *NPR1* gene (also called *NIM1* or *SAI1*), which was originally discovered as a key regulatory gene for activation of *PR-1* gene expression that functions downstream of SA in the SAR pathway (reviewed by van Wees *et al*., 2000). In *Arabidopsis*, the cytoplasmic-located NPR1 and WRKY70 (a component downstream of NPR1) mediates the cross-talk between the SA and the JA-signaling pathways. WRKY70 is activated by SA but repressed by JA, possibly functioning as a signal integrator from the mutually antagonistic SA and JA pathways (reviewed by Garcion and Métraux, 2006). Extensive genomic studies about SAR in some plant species and particularly in *Arabidopsis thaliana*, showed that the SAR pathway is a complex network (Shah, 2003; Garcion and Métraux, 2006). Thus, in generating systemic resistance, plants may employ multiple signals of different compounds such as SA, JA, ET or NO. Studies with *Catharanthus roseus* plants or cell cultures showed that biotic- or abiotic stress could lead to the production of different secondary metabolites as a defense response, which might employ different signal compounds (reviewed in Chapter 3).

In the plant defense, SA thus plays a key role. However, the biosynthesis of SA is still a matter of debate; several biosynthetic pathways exist in nature (see Chapter 6). For many years it was thought that in plants SA was derived from phenylalanine via benzoic acid. However, Verberne *et al*. (2000) showed that it is possible to introduce the microbial SA biosynthesis via the isochorismate pathway in plants. Wildermuth *et al*. (2001) showed the involvement of the isochorismate synthase (ICS) gene in

Arabidopsis in the biosynthesis of SA, but so far no direct chemical evidence exists for this pathway in plants. Budi Muljono *et al*. (2002) showed that in *C. roseus* the closely related dihydroxybenzoic acid is derived from isochorismate by a retrobiosynthetic study. As this plant cell culture also produces small amounts of SA after elicitation, this cell culture seemed an excellent model for studying the SA biosynthesis.

Labeling experiments with a stable ^{13}C isotope are commonly used to map metabolic pathways since ^{13}C is not radio-active and nuclear magnetic resonance spectrometry (NMR) analysis allows determining the precise site of the label in a molecule. Natural abundance of ^{13}C is 1.1%, so on a labeling of 1.1% will lead to a doubling of the percentage of the carbon being labeled and consequently to a clear increase of the signal concerned. In this way relative labeling of all carbons in a molecule can be measured. A high-level of incorporation of the label is important for a successful of labeling experiment. This is determined by several factors such as plant species, kind of labeled-precursor administered (the number of potential metabolic steps for converting the administered precursor to the target compound), the level of the precursor in the medium, kind- and amount of the cells, the activation of the target pathway (due to biotic- or abiotic stresses), the metabolic stability of the target compound, etcetera. Early precursors such as [1-^{13}C]-D-glucose or [U-^{13}C]-D-glucose are often used for the study of a biosynthetic pathway in yeast or plants (e.g. Werner *et al*., 1997). *Catharanthus roseus* suspension cultures have been shown to be a suitable model for retrobiosynthetic studies of iridoids (Contin *et al*., 1998) and 2,3-DHBA (Budi Muljono *et al*., 2002).

Aim of the thesis

The aim of this study is to map the biosynthetic pathway of salicylic acid in *C. roseus* cell suspension culture elicited by *Pythium aphanidermatum* extract using a retrobiosynthetic approach.

Outline of the thesis

SA is a C6C1 compound derived from chorismate either via the precursor phenylalanine or isochorismate. A review about chorismate-derived C6C1 compounds with the emphasis on the biosynthetic pathways is presented in **Chapter 2**. SA belongs to the phenolic compounds, a group of secondary metabolites that is widely

distributed in plants and often their production is increased under stress conditions. The phenolic compounds present in *C. roseus*, their biosynthetic pathways and regulation are discussed in **Chapter 3**. The level of SA in some lines of *C. roseus* cell suspension cultures after elicitation with *Pythium* extract were studied (**Chapter 4)** in order to select a high-SA producing cell line that would be used as a model in the labeling experiment. **Chapter 5** deals with development of a purification method of SA to allow analysis of the trace amonts in the cells by NMR. The results of the labeling experiments using the early-precursor [1-^{13}C]-D-glucose are reported in **Chapter 6**. SA is an important signaling compound in SAR, the effect of exogenous SA on the metabolites in a *C. roseus* suspension cells during a time course is reported in **Chapter 7**. Finally, a summary is presented at the end of the thesis.

Chapter 2

Chorismate-derived C6C1 compounds in plants

Published in *Planta* (2005) 222: 1-5

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Keywords: chorismate-derived C6C1 compounds, biosynthesis, plants

2.1 Introduction

The secondary metabolites are the products of interaction of the producing organism with its environment and have a restricted occurrence. Many have economical importance as, e.g. drugs, antioxidants, flavors, fragrances, dyes, insecticides and pheromones (Verpoorte *et al*., 2002). Secondary metabolites can be classified according to their biosynthetic building blocks or their carbon skeleton. The C6C1 compounds are compounds having an aromatic six-carbon ring with one carbon attached. They are generally derived from the shikimate pathway (Dewick, 2002).

The shikimate pathway, restricted to microorganisms and plants, includes seven metabolic steps, starting with phosphoenolpyruvate and D-erythrose-4-phosphate, and ending with chorismate (an important metabolic branch-point) (Figure 2.1). All enzymes involved have been purified and the cDNAs characterized from some prokaryotes and eukaryotes (Herrmann and Weaver, 1999). In plants, the pathway is localized in plastids.

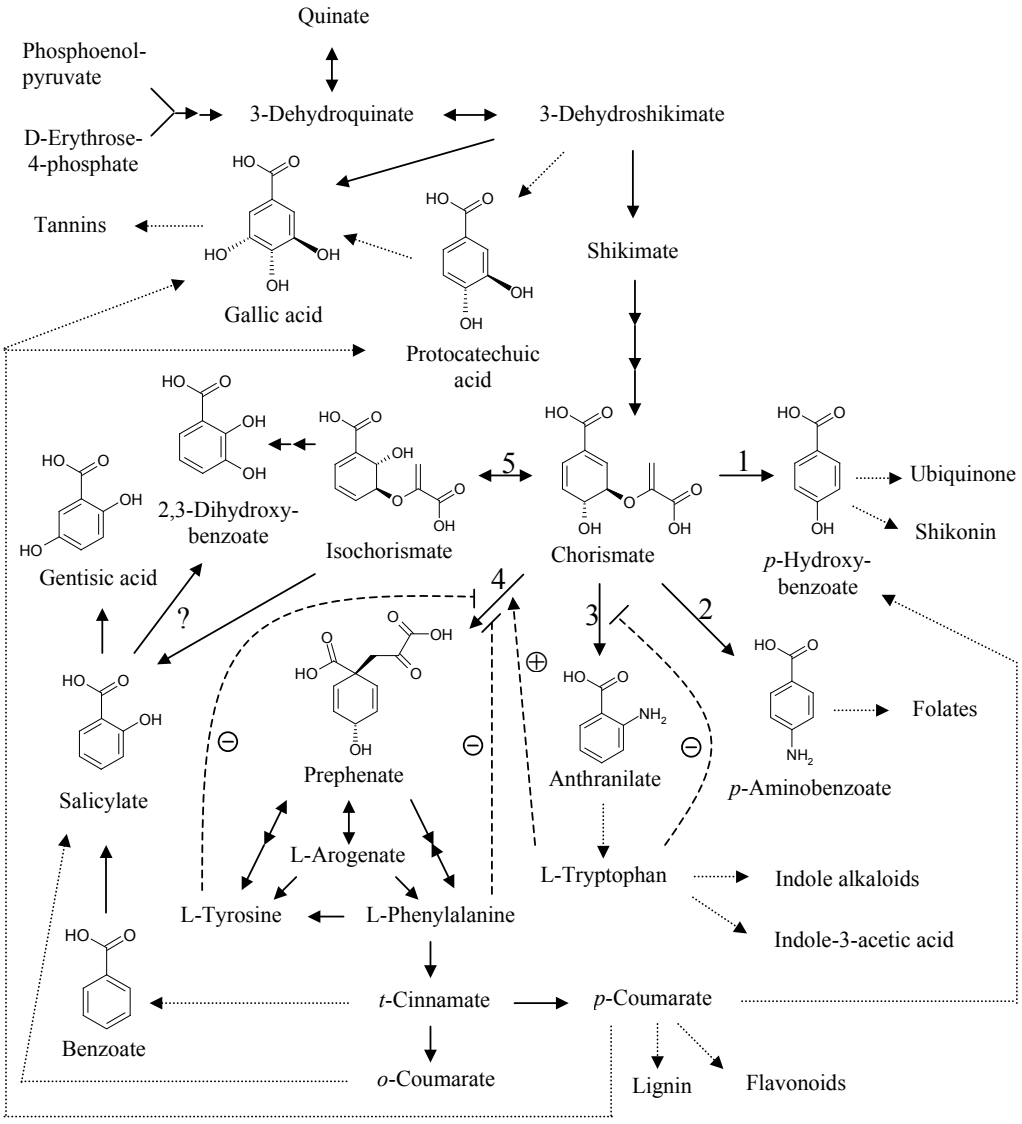

Figure 2.1 The biosynthetic pathway of chorismate/isochorismate derived-C6C1 compounds. 1 = chorismate pyruvate-lyase; 2 = *p*-aminobenzoate synthase; 3 = anthranilate synthase; 4 = chorismate mutase; 5 = isochorismate synthase. The dashed lines with + and – indicate feedback activation and inhibition respectively. A dotted line means multi-step reactions.

Gallic acid and protocatechuic acid (3,4-dihydroxybenzoic acid) are C6C1 compounds that can derive from either shikimate pathway (by dehydration and dehydrogenation of 3-dehydroshikimic acid) or phenylalanine pathway (via 3,4,5-trihydroxycinnamic acid) (Torssell, 1997; Ossipov *et al*. 2003). Gallic acid can also derive from orsellinic acid via the polyketide pathway by decarboxylation and oxidation (Torssell, 1997). This review will focus on chorismate-derived C6C1 compounds in plants, including anthranilate, *p*-aminobenzoate, *p*-hydroxybenzoate, salicylate and 2,3-dihydroxybenzoate.

2.2 Anthranilate

Anthranilate is the product of anthranilate synthase (AS, EC 4.1.3.27), the first enzyme of the tryptophan biosynthesis. The flux through this pathway is controlled by feedback inhibition by tryptophan on AS (Li and Last, 1996). AS is a key regulator for alkaloid accumulation induced by elicitors in *Ruta graveolens* (Bohlmann *et al*., 1995) and it may be a rate-limiting enzyme in the biosynthesis of avenanthramides, indole phytoalexins in oats (Matsukawa *et al*., 2002).

AS holoenzymes are characterized as tetramers consisting of two α- and two β-subunits, encoded by separate nuclear genes, synthesized in the cytosol and transported into the plastid to obtain the mature active form (Zhang *et al*., 2001). Two genes encoding ASα subunits (*ASA1* and *ASA2*) were isolated from *Arabidopsis thaliana* and found to be functional by complementation in yeast and *E. coli* (Niyogi and Fink, 1992). The overexpression of the *Ruta graveolens* ASα isozymes in *E. coli* revealed the presence of a tryptophan feedback-insensitive ASα1 and a sensitive ASα2 enzyme (Bohlmann *et al*., 1996). Transformation of a 5-methyl tryptophan-resistant tobacco gene (*ASA2*) into *Astragalus sinicus* (a forage legume) resulted in an increased level of tryptophan (Cho *et al*., 2000). An *Arabidopsis* feedback-resistant ASα gene (a mutated *ASA1*) was transformed into *Catharanthus roseus* providing hairy roots with increased levels of tryptophan, tryptamine and the indole alkaloid lochnericine (Hughes *et al*., 2004). Relocating a native tryptophan feedback-insensitive gene from the nucleus to the plastid genome resulted in transplastomic tobacco plants with greatly increased tryptophan levels but normal phenotype and fertility, showing the advantage of plastid transformation compared to nuclear transformation (Zhang *et al*., 2001). Replacing aspartate with asparagine at a certain

position in *A. thaliana* (Li and Last, 1996) or *Oryza sativa* ASα (Tozawa *et al*., 2001), resulted in lower sensitivity for tryptophan inhibition. Sensitivity for tryptophan inhibition can also be due to a mutation in a regulator gene of the AS gene's expression (Ishikawa *et al*., 2003). The genes encoding the rice plastidial ASβ subunits have been characterized (Kanno *et al*., 2004). Both ASβ subunits are assembled with the mature forms of the ASα subunits.

2.3 Salicylic acid

Salicylic acid (SA) has several roles in plants (Raskin, 1992) including the induction of systemic acquired resistance (SAR) as response to pathogens. SA-dependent SAR is characterized by the increase of SA and its conjugates and pathogenesis related (PR) proteins (Ryals *et al*., 1996).

SA in plants is thought to be derived from the phenylalanine pathway by cinnamic acid chain shortening, either through a β-oxidative or a non-oxidative pathway. Some steps have been identified, others not yet (Verberne *et al*., 1999). The enzyme (benzoic acid 2-hydroxylase) converting benzoic acid (BA) into SA has been identified (Leon *et al*., 1995). The non-oxidative pathway to BA does not function in cucumber (*Cucumis sativus*) and *Nicotiana attenuata* (Jarvis *et al*., 2000). In microorganisms, SA biosynthesis involves isochorismate synthase (ICS, EC 5.4.99.6), converting chorismate into isochorismate, and isochorismate pyruvate lyase (IPL) providing SA (reviewed by Verberne *et al*., 1999). Verberne *et al*. (2000) suggested that plants may utilize this pathway and they introduced the microbial-isochorismate SA pathway into tobacco resulting in increased-SA levels and enhanced resistance to tobacco mosaic virus. Wildermuth *et al*. (2001) found evidence for a SA isochorismate pathway. The *Arabidopsis sid2* mutant unable to produce chloroplast-localized ICS1 exhibited a remarkable lower level of SA after infection and a reduced resistance against pathogens. Chong *et al*. (2001) showed that the SA accumulation in elicited tobacco cells required de novo BA synthesis from *trans*-cinnamic acid, though, instead of BA, the benzoyl-glucose was the likely intermediate. The pathway from *trans*-cinnamic acid to SA via BA is involved in the stress-induced flowering of *Pharbitis nil* (Hatayama and Takeno, 2003).

The catabolism of SA is mainly through glucosylation by SAglucosyl transferase, which occurs presumably in the cytoplasm and subsequently accumulated in the

vacuoles. The uptake of SAG into vacuoles may involve different mechanisms in different plant species. For example, in soybean (*Glycine max*), the ATP-binding cassette (ABC) transporter is involved, whereas in the red beet it is the H^+-antiport mechanism (Dean and Mills, 2004). In a *Catharanthus roseus* cell suspension culture, SA was catabolized by a hydroxylation into 2,5-dihydroxybenzoic acid (gentisic acid) followed by a glucosylation of the newly introduced phenolic hydroxyl group. The 55 kDa hydroxylase and the 41 kDa regiospecific glucosyltransferase have been isolated by Shimoda *et al*. (2004) and Yamane *et al*. (2002).

2.4 2,3-Dihydroxybenzoate

2,3-Dihydroxybenzoate (2,3-DHBA) is in microorganisms derived from isochorismate (Young *et al*., 1968). SA and 2,3-DHBA are precursors of siderophores such as enterobactin and pyocheline. This pathway involves ICS, 2,3-dihydro-2,3-DHBA synthase and 2,3-dihydro-2,3-DHBA dehydrogenase. 2,3-DHBA may derive from SA by hydroxylation (reviewed by Budi Muljono, 2002). 2,3-DHBA is produced in *Catharanthus roseus* cell cultures after elicitation with fungal cell-wall preparations and parallels an increase in activity of ICS (Moreno *et al*. 1994). The ICS protein and its cDNA were obtained from *C. roseus* cell cultures (van Tegelen *et al*., 1999). This ICS has 57% homology with the ICS1 of *A. thaliana* and 20% homology with bacterial ICS (Wildermuth *et al*., 2001). A retrobiosynthetic study with *C. roseus* suspension cells fed with [1-^{13}C]glucose confirmed the intermediacy of isochorismate in 2,3-DHBA biosynthesis (Budi Muljono *et al*., 2002).

2.5 *p*-Hydroxybenzoate

p-Hydroxybenzoate (4HB), a precursor of shikonin, is formed via the phenylpropanoid pathway (Löscher and Heide, 1994). It is also a precursor of ubiquinones formed directly from chorismate by chorismate pyruvate-lyase (CPL) in bacteria or from both pathways in eukaryotic microorganisms (Meganathan, 2001). The *ubiC* gene encoding CPL of *E. coli* was overexpressed in tobacco resulting in high CPL activity and increased level of 4HB as β-glucosides (4HBG, 0.52% DW) derived from the introduced pathway (Siebert *et al*., 1996). Using the same constructs, only 20% of the total 4 HBG produced in *Lithospermum erythrorhizon* employed this pathway (Sommer *et al*., 1999). Transformation using a strong $(ocs)_3$mas promoter

did not change the level of 4HBG compared to the control cultures, but 73% of total 4HBG was derived from the introduced pathway (Köhle *et al*., 2002). Whilst, introducing this construct into tobacco and potato led to 5.1% (DW) of 4HBG in tobacco cell cultures and 4.0% DW in the leaves of potato shoots. These amounts correlated with CPL activity and are the highest for artificial secondary metabolites ever reached by genetic engineering in plants. It did not affect growth, proving the large capacity of the plastidial shikimate pathway (Köhle *et al*., 2003). *UbiC* without a transit peptide provided much lower levels of 4HB derivatives (Sommer and Heide, 1998). In *L. erythrorhizon*, 4HBG was accumulated in vacuoles. The vacuolar transport of 4HB and of *p*-hydroxycinnamic acid in red beet requires glucosylation and employs an H^+-antiport mechanism, the same transport used by 5-hydroxychlorsulphuron (a herbicide)-glucoside (Bartholomew *et al*., 2002).

2.6 *p*-Aminobenzoate

p-Aminobenzoate (PABA) is the precursor of folic acids (folates). Folates are cofactors in "one carbon" transfer reactions as e.g. in the biosynthesis of some nucleotide bases (Scott *et al*., 2000). The conversion of chorismate into PABA in microorganisms is catalyzed by *p*-aminobenzoic acid synthase, EC 4.1.3.-. This enzyme consists of three subunits. The large subunit (aminodeoxychorismate synthase) encoded by *pabB*, converts chorismate into aminodeoxychorismate (ADC), the small subunit encoded by *pabA* is a glutamine amidotransferase and the third subunit (aminodeoxychorismate lyase) encoded by *pabC*, converts ADC into PABA and pyruvate (Viswanathan *et al*., 1995).

Sulfonamides are PABA analogues inhibiting dihydropteroate synthase (DHPS), the enzyme converting PABA into 7,8-dihydropteroate (Scott *et al*., 2000). DHPS is the key regulator of the folate biosynthetic pathway (Mouillon *et al*., 2002). The cDNA was recently purified and characterized from pea leaves. The presence of a putative mitochondrial transit peptide of 28 amino acids in the single copy gene, indicates the mitochondria as the site of 7,8-dihydropteroate synthesis (Rebeille *et al*., 1997), thus requiring transport of PABA across the plastidial- and mitochondrial membranes.

2.7 Conclusion

One should be very careful in extrapolating findings of C6C1 pathways in a plant e.g. *Arabidopsis* to other plants. It can not be excluded that particularly for secondary metabolites different localization and regulation of the pathways occurs in different plant species. Chorismate is biosynthesized in plastids, where also most of the enzymes discussed are localized. But chorismate may be transported out of plastids and further converted in other compartments. For example, plants overexpressing microbial SA genes without plastidial signal sequence still produced small amounts of SA, thus requiring transport of chorismate. AS has also been proposed to have a plastidial and a cytosolic form, though evidence is lacking. The flux through the different branches is quite different with the chorismate mutase (CM) pathway generally being the most active. Unraveling all the C6C1 pathways on the level of genes, proteins and intermediates including localization (transport) and regulation will be a major challenge for the coming years.

Chapter 3

Phenolic compounds in *Catharanthus roseus*

Accepted in *Phytochemistry Reviews* (2006)

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Abstract

Besides alkaloids *Catharanthus roseus* produces a wide spectrum of phenolic compounds, this includes C6C1 compounds such as 2,3-dihydroxybenzoic acid, as well as phenylpropanoids such as cinnamic acid derivatives, flavonoids and anthocyanins. The occurrence of these compounds in *C. roseus* is reviewed as well as their biosynthesis and the regulation of the pathways. Both types of compounds compete with the indole alkaloid biosynthesis for chorismate, an important intermediate in plant metabolism. The biosynthesis of C6C1 compounds are induced by biotic elicitors.

Keywords: phenolic compounds, *Catharanthus roseus*

3.1 Introduction

Plant phenolics cover several groups of compounds such as simple phenolics, phenolic acids, flavonoids, isoflavonoids, tannins and lignins since they are defined as compounds having at least one aromatic ring substituted by at least one hydroxyl group. The hydroxyl group(s) can be free or engaged in another function as ether, ester or glycoside (Bruneton, 1999). They are widely distributed in plants and particularly present in increased levels, either as soluble or cell wall-bound

compounds, as a result of interaction of a plant with its environment (Matern *et al*., 1995).

Catharanthus roseus (L.) G.Don (Madagascar periwinkle) is a terpenoid indole alkaloids (TIAs) producing plant. In attempts to improve the production of the valuable alkaloids such as vincristine and vinblastine, several studies on *C. roseus* reported also the accumulation of phenolic compounds upon biotic and/or abiotic stress. The accumulation of phenolics may also affect other secondary metabolite pathways including the alkaloid pathways, as plant defense is a complex system. Elucidation of the pathways and understanding their regulation are important for metabolic engineering to improve the production of desired metabolites (Verpoorte *et al*., 2002). This review deals with the phytochemistry of phenolic compounds in *C. roseus*, their biosynthesis and its regulation.

3.2 Phytochemistry

Simple phenolics are termed as compounds having at least one hydroxyl group attached to an aromatic ring, for example catechol.

Most compounds having a C6C1 carbon skeleton, usually with a carboxyl group attached to the aromatic ring (Dewick, 2002), are phenolics. C6C1 compounds in *C. roseus* include benzoic acid (BA) and phenolic acids derived from BA e.g. *p*-hydroxybenzoic acid (*p*-HBA), salicylic acid (SA), 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA), 3,4-dihydroxybenzoic acid (3,4-DHBA), 3,5-dihydroxybenzoic acid (3,5-DHBA), gallic acid (GA) and vanillic acid.

Simple phenylpropanoids are defined as secondary metabolites derived from phenylalanine, having a C6C3 carbon skeleton and most of them are phenolic acids. For example: *t*-cinnamic acid, *o*-coumaric acid, *p*-coumaric acid, caffeic acid and ferulic acid. A simple phenylpropanoid can conjugate with an intermediate from the shikimate pathway such as quinic acid to form compounds like chlorogenic acid.

Compounds having a C6C3C6 carbon skeleton such as flavonoids (including anthocyanins) and isoflavonoids, are also among the phenolic compounds in *C. roseus*.

The C6C1-, C6C3- and C6C3C6 compounds reported to be present in *C. roseus* are reviewed in Table 3.1.

Table 3.1. Phenolic compounds in *Catharanthus roseus*

Compound's name	Plant material	Analytical method	Reference
C6C1 :			
2,3-DHBA	Cell suspension culture Cell suspension culture Cell suspension culture Cell suspension culture	RP-HPLC Capillary GC ^{13}C-NMR; MS RP-HPLC	Moreno *et al*., 1994a. Budi Muljono *et al*., 1998 Budi Muljono *et al*., 2002 Talou *et al*., 2002.
2,3-DHBAG	Cell suspension culture	RP-HPLC	Budi Muljono *et al*. 2002; Talou *et al*., 2002
SA	Cell suspension culture	Capillary GC	Budi Muljono *et al*., 1998
SA; SAG	Cell suspension culture Cell suspension culture	RP-HPLC RP-HPLC; IEC-^{1}H-NMR; ^{13}C-NMR	Budi Muljono, 2001. Mustafa *et al*., unpublished results.
Benzoic acid	Cell suspension culture	Capillary GC	Budi Muljono *et al*., 1998
2,5-DHBA	Cell suspension culture	Capillary GC	Budi Muljono *et al*., 1998
2,5-DHBA; 2,5-DHBAG	Cell suspension culture	Preparative TLC; GLC FAB-MS; NMR	Shimoda *et al*., 2002; Yamane *et al*., 2002; Shimoda *et al*., 2004.
Gallic acid	Plant	RP-HPLC	Proestos *et al*., 2005.
Glucovanillin	Cell suspension culture	RP-HPLC	Sommer *et al*., 1997; Yuana *et al*., 2002.
Vanillic acid	Plant Cell suspension culture	RP-HPLC RP-HPLC	Proestos *et al*., 2005. Yuana *et al*., 2002.
Glucovanillic acid	Cell suspension culture	RP-HPLC	Yuana *et al*., 2002.
Vanillyl alcohol	Cell suspension culture	RP-HPLC	Sommer *et al*., 1997; Yuana *et al*., 2002.
Vanillyl alcohol-phenyl-glucoside	Cell suspension culture	RP-HPLC	Sommer *et al*., 1997; Yuana *et al*., 2002.
C6C3 / conjugated C6C3:			
t-Cinnamic acid	Cell suspension culture Cell suspension culture	RP-HPLC Capillary GC	Moreno, 1995. Budi Muljono *et al*., 1998
Hydroxytyrosol	Plant	RP-HPLC	Proestos *et al*., 2005.
Ferulic acid	Plant	RP-HPLC	Proestos *et al*., 2005.
Chlorogenic acid	Leaves	^{1}H-NMR	Choi *et al*., 2004.
C6C3C6 / conjugated C6C3C6:			
Kaemferol	Flower	Paper chromatography (PC)	Forsyth and Simmonds, 1957.

Kaemferol trisaccharides	Leaves	Column chromatography (CC); UV; MS; NMR	Nishibe *et al*., 1996.
	Stem	CC; UV; MS; NMR	Brun *et al*., 1999.
Quercetin	Flower	PC	Forsyth and Simmonds, 1957.
Quercetin trisaccharides	Leaves	CC; UV; MS; NMR	Nishibe *et al*., 1996.
Quercetin trisaccharides	Stem	CC; UV; MS; NMR	Brun *et al*., 1999.
Syringetin glycosides	Stem	CC; UV; MS; NMR	Brun *et al*., 1999.
Malvidin	Flower	PC	Forsyth and Simmonds, 1957.
	Callus culture	CC; PC; TLC; UV	Carew and Krueger, 1976.
	Cell suspension culture	PC; TLC; HPLC	Knobloch *et al*., 1982.
Malvidin 3-*O*-glucosides	Flowers & cell suspension cultures	ESI-MS/MS	Filippini *et al*., 2003.
Malvidin 3-*O*-(6-*O*-*p*-coumaroyl)	Flowers & cell suspension cultures	ESI-MS/MS	Filippini *et al*., 2003.
Petunidin	Flower	PC	Forsyth and Simmonds, 1957.
	Callus culture	CC; PC; TLC; UV	Carew and Krueger, 1976.
	Cell suspension culture	PC; TLC; HPLC	Knobloch *et al*., 1982.
Petunidin 3-*O*-glucosides	Flowers & cell suspension cultures	ESI-MS/MS	Filippini *et al*., 2003.
Petunidin 3-*O*-(6-*O*-*p*-coumaroyl)	Flowers & cell suspension cultures	ESI-MS/MS	Filippini *et al*., 2003.
Hirsutidin	Flower	CC	Forsyth and Simmonds, 1957.
	Callus culture	CC; PC; TLC; UV	Carew and Krueger, 1976.
	Cell suspension culture	PC; TLC; HPLC	Knobloch *et al*., 1982.
Hirsutidin 3-*O*-glucosides	Flowers & cell suspension cultures	ESI-MS/MS	Filippini *et al*., 2003.
Hirsutidin 3-*O*-(6-*O*-*p*-coumaroyl)	Flowers & cell suspension cultures	ESI-MS/MS	Filippini *et al*., 2003.

3.3 Biosynthesis

Phenolic compounds are generally synthesized *via* the shikimate pathway. Another pathway, the polyketide pathway, can also provide some phenolics e.g. orcinols and quinones. Phenolic compounds derived from both pathways are quite common e.g. flavonoids, stilbenes, pyrones and xanthones (Bruneton, 1999).

The shikimate pathway, a major biosynthetic route for both primary- and secondary metabolism, includes seven steps. It starts with phosphoenolpyruvate and erythrose-4-phosphate and ends with chorismate (Herrmann and Weaver, 1999). Chorismate is an important branching point since it is the substrate of 5 enzymes: chorismate mutase (CM, EC 5.4.99.5), isochorismate synthase (ICS, EC 5.4.99.6), *p*-hydroxybenzoate synthase or chorismate pyruvate-lyase, anthranilate synthase (AS, EC 4.1.3.27) and *p*-aminobenzoate synthase (EC 4.1.3.38.) (reviewed by Mustafa and Verpoorte, 2005). These enzymes are the starting points of several pathways leading to a great diversity of secondary metabolites including phenolics. For example, CM is responsible for the formation of prephenate, the first intermediate of phenylalanine biosynthesis. In plants, phenylalanine is thought to be the general precursor of C6C1-, C6C3- and C6C3C6 compounds and their polymers such as tannins and lignins (Wink, 2000). Figure 3.1 shows the biosynthetic pathway of some phenolics.

3.3.1 Biosynthesis of C6C1

In the phenylpropanoid pathway, *β*-oxidation of the propyl-moiety of a C6C3 results in a C6C1, the aromatic hydroxylation generally occurs more effectively at the C6C3 level than at the C6C1 level (Torsell, 1997). However, it has been shown in some studies that C6C1 gallic acid and the related hydrolysable tannins are synthesized from an early intermediate of the shikimate pathway rather than from phenylalanine or tyrosine (Werner *et al*., 1997; Ossipov *et al*., 2003). Löscher and Heide (1994) showed that *p*-HBA is derived from the phenylalanine pathway, though it has been proposed that the presence of the chorismate pathway leading to this compound in plants is highly probable. Other C6C1 compounds such as SA and 2,3-DHBA were proven in some plants to be synthesized *via* the isochorismate pathway (Wildermuth *et al*., 2001; Budi Muljono *et al*., 2002; Chapter 6 of this thesis). In microorganisms, isochorismate is a precursor of SA and 2,3-DHBA. Both are precursors of pyochelin and enterobactin, chelating agents needed by the host for

survival in an environment lacking soluble iron (Fe^{3+}) (reviewed by Verberne *et al*., 1999).

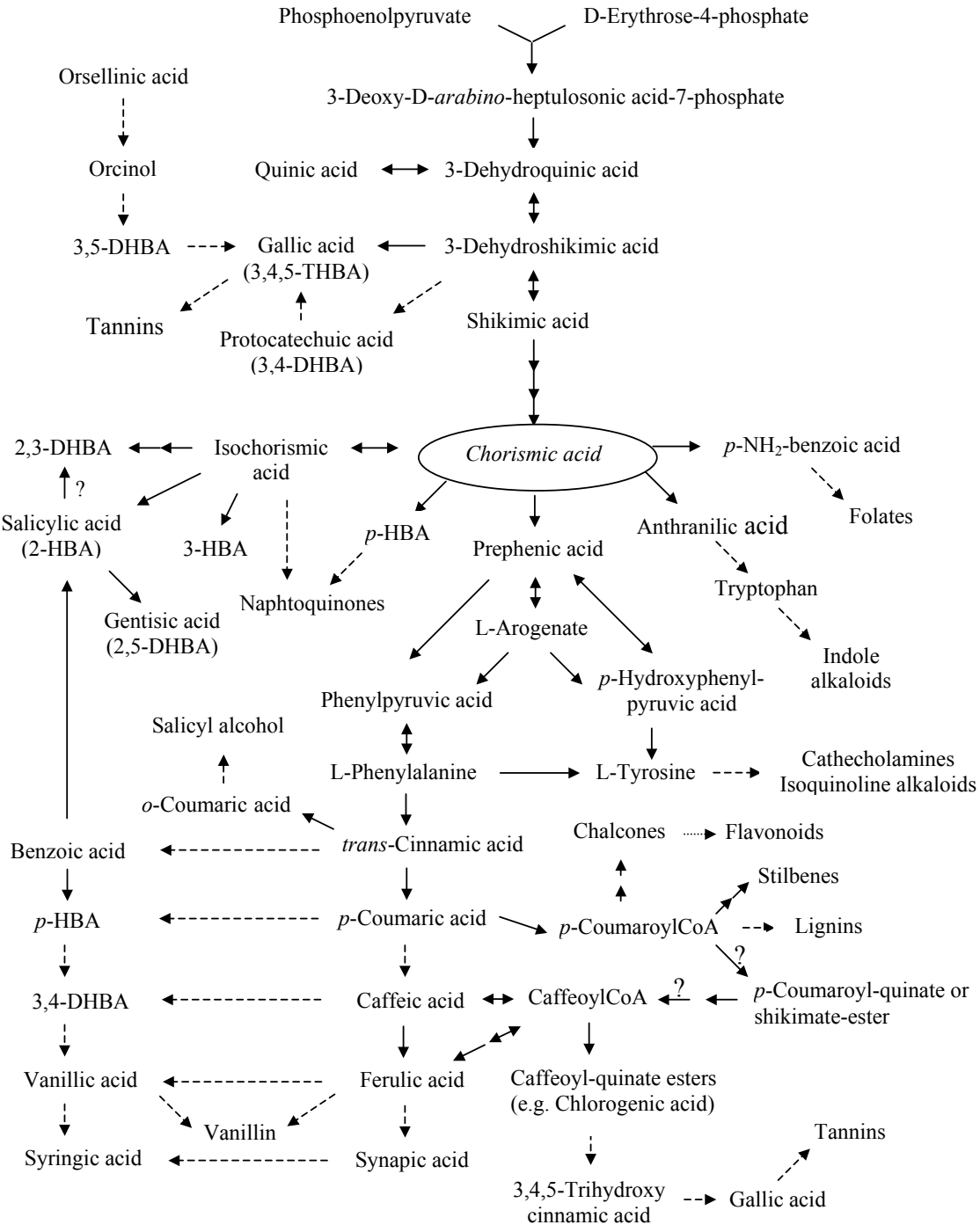

Figure 3.1. The biosynthetic pathway of some phenolic compounds. A small-dashed line means multi-steps reactions.

ICS is the enzyme responsible for conversion of chorismate into isochorismate. In *C. roseus*, the ICS activity was first detected in protein extracts of the cell cultures (Poulsen *et al*., 1991). Its activity increased after elicitation with fungal (*Pythium aphanidermatum*) extract, resulting in the production of 2,3-DHBA (Moreno *et al*., 1994a). The purification of this enzyme showed the presence of two isoforms, which require Mg^{2+} for enzyme activity and are not inhibited by aromatic amino acids. Isolation of its cDNA revealed the existence of only one ICS gene in this plant encoding a 64 kD protein with an N-terminal chloroplast-targeting signal. The deduced amino acid sequence shares homology with bacterial ICS and also with AS from plants (van Tegelen *et al*., 1999).

Some constructs containing a *C. roseus* cDNA clone of *ics* in sense or antisense orientation were successfully transformed into the *C. roseus* CRPM cell line (grown in Murashige & Skoog/ M&S medium with growth hormones), whereas the transformation into A12A2 line (grown in M&S medium without growth hormones) failed (Talou *et al*., 2001). Analysis of enzyme activities of ICS, AS and CM of the *ics*-sense line showed an increased (about 2-fold) ICS activity, a relatively non-altered AS activity and inhibition of CM activity. However, the *ics*-antisense line revealed that there was no correlation between *ics*-mRNA transcription and ICS activity, since it produced a lower level of *ics*-mRNA but a comparable level of ICS activity compared with that of the line transformed with an empty vector after elicitation. Also, the ICS activity was similar for the non-elicited *ics*-sense line and the elicited empty vector line though the latter produced a much higher level of the mRNA. After elicitation, 2,3-DHBA was not detectable in the cells or medium of either CRPM wild type or empty vector line. Surprisingly, the *ics*-antisense line provided a higher level of 2,3-DHBA in the cells than the *ics*-sense line with or without elicitation, whereas much lower levels of this compound were found in the medium of both cultures. Wild type A12A2 elicited cells produced much higher level of 2,3-DHBA compared with *ics*-sense- and *ics*-antisense elicited or non-elicited cells. The presence of the growth hormones in the medium might also affect enzymatic steps downstream of ICS, which is rate limiting for either 2,3-DHBA or SA accumulation in the CRPM line (Talou *et al*., 2001).

A retrobiosynthetic study of 2,3-DHBA in *C. roseus* showed that the ICS pathway was responsible for the increased level of this compound after elicitation (Budi Muljono *et al*., 2002). The ICS pathway leading to 2,3-DHBA includes ICS, 2,3-

dihydro-2,3-dihydroxybenzoate synthase for removing the enolpyruvyl side chain of isochorismate and 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase for the oxidation of 2,3-dihydro-DHBA to 2,3-DHBA (Young *et al*., 1969).

Besides 2,3-DHBA, Budi Muljono *et al*. (1998) reported the presence of SA in *C. roseus* cell cultures. SA plays different roles in plants (Raskin 1992), the most important is as signaling compound in systemic acquired resistance (SAR) (Ryals *et al*., 1996; Dempsey *et al*., 1999). Many studies dealing with SA-dependent- and/or SA-independent pathways in plant defense response have been performed in different plant species (particularly in *Arabidopsis*) showing the complexity of the SAR network (Shah, 2003). In microorganisms, the isochorismate pathway leading to SA involves ICS and isochorismate pyruvate-lyase (IPL). In plants, SA is thought to be derived from the phenylalanine pathway by chain shortening of a hydroxycinnamic acid derivative leading to BA. The complete pathway has not been resolved yet, though the enzyme responsible for the last step, converting BA to SA, has been characterized (Leon *et al*., 1995). In *Arabidopsis*, the enzyme ICS1 seems to be responsible for SA synthesis in SAR, it shares 57% homology with ICS from *C. roseus* (Wildermuth *et al*., 2001).

Since the ICS pathway leading to 2,3-DHBA exists in *C. roseus*, the existence of the ICS pathway leading to SA in the same plant is also possible. Verberne *et al*. (2000) proposed the presence of the ICS pathway leading to SA in plants. Both the ICS and phenylalanine pathways may occur in *C. roseus* and may be regulated differently for different functions as it was proposed by Wildermuth *et al*. (2001) with *Arabidopsis*. The latter group found that *Arabidopsis sid2-2* mutant, unable to produce ICS1, showed increased-susceptibility for pathogens, though it still produced a small amount of SA. However, the function and regulation of two pathways can be different in each species since Chong *et al*. (2001) showed that the SA accumulation in elicited tobacco cells required de novo BA synthesis from *trans*-cinnamic acid.

Glucosylation is found to be a rapid and main catabolic route for SA in several plants, providing β-*O*-D-glucosylsalicylic acid and/or SA glucose ester (e.g. Lee and Raskin, 1998; Dean and Mills, 2004). Increased level of SA glucoside (SAG) in *C. roseus* A12A2- and A11 (grown in Gamborg B5 medium with 1-naphtaleneacetic acid/ NAA) cells occurred after fungal elicitation (chapter 4 of this thesis), whereas a lower amount of SAG was detected in the CRPM cell line. A glycoside of SA, 3-β-*O*-

D-glucopyranosyloxy-2-hydroxybenzoic acid, was isolated from the leaves of *Vinca minor* L. (Nishibe *et al*., 1996).

In plants, 2,3-DHBA and 2,5-DHBA may also derive from SA. The roles of these compounds in plants are still not clear and it was thought that they are the products of metabolic inactivation by additional hydroxylation of the aromatic ring (El-Basyouni *et al*., 1964; Ibrahim and Towers, 1959). Besides SA and 2,3-DHBA, the other C6C1 compounds such as BA and 2,5-DHBA were detected in a *C. roseus* cell suspension culture by capillary GC (Budi Muljono *et al*., 1998).

Shimoda *et al*. (2002) showed that in *C. roseus* cells grown in Schenk and Hildebrandt (SH) medium with 10 mM 2,4-dichlorophenoxyacetic acid (2,4-D), SA was catabolized by a hydroxylation into 2,5-DHBA (gentisic acid) followed by a glucosylation of the newly introduced phenolic hydroxyl group. The glucosyltransferase specific for gentisic acid was isolated from *C. roseus* cell cultures (Yamane *et al*., 2002). This 41 kDa protein is regioselective, transferring glucose from UDP-glucose onto the oxygen atom of the 5-hydroxyl group of this compound. It worked also for 7-hydroxyl groups of hydrocoumarins though the relative activities were low (< 1.2%) compared to that for 5-hydroxyl group of gentisic acid. Optimum activity was at pH 8.0 and the enzyme was strongly inhibited by divalent cations such as Mn^{2+}, Co^{2+}, Zn^{2+} and Fe^{2+}. Shimoda *et al*. (2004) isolated a novel 55 kDa hydroxylase from *C. roseus* cell cultures which is responsible for the hydroxylation of SA into gentisic acid. The enzyme activity was optimal at pH 7.8 and was completely inhibited by divalent cations such as Cu^{2+} and Hg^{2+}.

Catharanthus roseus cell suspension culture was reported to be able to accumulate high amount of glucovanillin after 16 h incubation time with 8.2 mM of vanillin (Sommer *et al*., 1997). Besides, some other C6C1 compounds such as vanillyl alcohol and vanillyl alcohol-phenyl glucoside were also found as the reduction products of vanillin and glucovanillin. Observation after 12 h and 24 h feeding experiment of a *C. roseus* suspension culture with vanillin showed that 12 h incubation and a cell density of 10 g inoculum provided the highest amount (16% conversion) of glucovanillin (Yuana *et al*. 2002). The levels of vanillin and glucovanillin decreased after 24 h. The *C. roseus* suspension cultures were grown in M&S medium containing growth hormones (1 mg/L 2,4-D and 1 mg/L kinetin). Besides the reduction products as mentioned by Sommer *et al*. (1997), this group reported also the presence of other

C6C1 compounds such as vanillic acid and its glucosides (glucovanillic acid). The presence of vanillic acid in *C. roseus* plant was reported by Proestos *et al*. (2005).

3.3.2 Biosynthesis of C6C3

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), responsible for the conversion of phenylalanine into cinnamic acid, is the entry-point enzyme into the phenylpropanoid pathways since the reaction product is a precursor for several phenylpropanoids for example, the simple phenylpropanoids (C6C3 compounds) such as cinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid. Besides the precursors of C6C1 compounds, simple phenylpropanoids are also precursors of other phenolics, which in many plants act as phytoalexins or phytoanticipins e.g. flavonoids, isoflavonoids, stilbenes, monolignols and lignans (Dixon, 2001), or as physical barrier against pathogen infiltration e.g. the phenylpropanoid polymer: lignin (Boudet *et al*., 1995, Mitchell *et al*., 1999). Activation of PAL is considered as a marker for ongoing SAR in a plant.

By capillary gas chromatography (GC), the presence of *trans*-cinnamic acid was detected in an extract of a *C. roseus* cell suspension culture (Budi Muljono *et al*., 1998). A reversed phase high performance liquid chromatography (RP-HPLC) analysis of phenolic compounds in some plant extracts showed that the *C. roseus* extracts contained the highest amount of a C6C3 hydroxytyrosol (310mg/100g DW) and a C6C1 gallic acid (42mg/100g DW) if compared to 26 other plant extracts analyzed. Other phenolics detected from this plant extract were ferulic acid (250mg/100g DW) and vanillic acid (1.3 mg/100g DW). No flavonoids were detected in this study (Proestos *et al*., 2005).

Cinnamate 4-hydroxylase (C4H), a cytochrome P_{450}-dependent enzyme, is responsible for the hydroxylation at the C-4 position of cinnamic acid to form *p*-coumaric acid. Hotze *et al*. (1995) isolated the cDNA of C4H of *C. roseus*. The enzyme shared 75.9% identity with C4H from other plants and the transcription was induced under various stress conditions.

Using ^{1}H-NMR spectroscopy and multivariate data analysis, Choi *et al*. (2004) found that increased levels of some phenolic compounds such as chlorogenic acid and polyphenols together with increased levels of some other metabolites were major discriminating factors between healthy- and phytoplasma-infected *C. roseus* leaves. The other metabolites present in increased levels were loganic acid, secologanin and

vindoline (from TIA pathway), succinic acid, glucose and sucrose. Some proton signals were detected close to those of chlorogenic signals (shifted approximately 0.05 ppm downfield), which are assumed to be other chlorogenic acid isomers such as 4-*O*-caffeoylquinic acid or 5-*O*-caffeoylquinic acid (Choi *et al*., 2004). These conjugated phenylpropanoids could be the products of an enzyme catalyzing the synthesis of quinate ester from caffeoyl-CoA. Caffeoyl-CoA and *p*-coumaroyl-CoA in tobacco, are the best acyl group donors for shikimate and quinate (acceptors) for the reaction catalyzed by hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyl-transferase (Hoffmann *et al*., 2003). This enzyme is important for the pathway leading to 3,4-dihydroxy substituted compounds, since in *Arabidopsis thaliana* it has been demonstrated that C-3 hydroxylation does not occur at the free acid level as in the case of C-4 hydroxylation. In this plant for example, *p*-coumarate 3-hydroxylase, a cytochrome-P_{450} enzyme, does not accept the free acid form or the *p*-coumaroyl-CoA ester, but only the shikimate and quinate esters of *p*-coumaroyl-CoA ester act as substrates providing caffeoyl-CoA and subsequently caffeic acid by a ligase (Schoch *et al*., 2001).

3.3.3 Biosynthesis of C6C3C6

A coupling of a *p*-hydroxycinnamoyl-CoA with three molecules of malonyl-CoA, subsequently followed by a Claisen-like reaction by a chalcone synthase, provides a chalcone. Chalcones are precursors for a wide range of flavonoid derivatives (C6C3C6 compounds). A Michael-type nucleophilic attack of the hydroxyl group on to the α,β-unsaturated ketone of a chalcone, leads to a flavanone (e.g. naringenin from naringenin-chalcone). From flavanones, several flavonoid groups are formed, e.g. flavones, flavonols, anthocyanidins and cathechins. The members of each group are distinguished due to the different hydroxylation patterns in the two aromatic rings, methylation, glucosylation and/or dimethylation. In plants, flavonoids occur mainly as water-soluble glycosides (Dewick, 2002).

The biosynthetic pathway of C6C3C6 leading to anthocyanins is one of the best-studied biosynthetic pathways in plants. One of the reasons is because dealing with colored compounds for analysis of mutants is relatively easy (reviewed by Verpoorte *et al*., 2002). However, so far there are not many studies about isolation of genes and enzymes involved in this pathway in *C. roseus*.

Some anthocyanidins and anthoxanthins in *C. roseus*, were first isolated from the fresh-petals by Forsyth and Simmonds (1957). Using acid-hydrolysis and separation on paper chromatography (PC), two minor anthocyanidins were identified as petunidin and malvidin. After a more complicated separation procedure employing acidic extraction, partitioning, column chromatography, re-extraction, precipitation and recrystallization, the major anthocyanidin was isolated and identified as hirsutidin. Two anthoxantins present in the flowers were identified as kaemferol and quercetin.

Nishibe *et al*. (1996) isolated two flavonoids: mauritianin (= kaemferol 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside) and quercetin 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside together with chlorogenic acid from the leaves of *C. roseus*. Whilst, from the leaves of *Vinca minor* they isolated a flavonoid kaemferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside-7-*O*-β-D-glucopyranoside together with 2,3-DHBA, 3-β-D-glucopyranosyloxy-2-hydroxybenzoic acid and chlorogenic acid. The two flavonoids isolated from the leaves of *C. roseus*, were also isolated from the stem by Brun *et al*., (1999). The latter group also isolated a new flavonol glycoside syringetin from this plant.

Filippini *et al*. (2003) developed a stable callus culture of *C. roseus* producing anthocyanins by continuous cell-aggregate selection. A stable cell suspension culture was obtained from this homogeneous red pigmentation calli (V32R), which contained 30% of cells accumulating anthocyanins. Similar anthocyanins were identified by ESI-MS/MS both in this cell suspension culture and in flowers of field-grown plants. They were identified as 3-*O*-glucosides and 3-*O*-(6-*O*-*p*-coumaroyl)glucosides of petunidin, malvidin and hirsutidin.

Methylations provide a variety of flavonoids including anthocyanins, which play a role in flower colors (Harborne and Williams, 2000). Two cDNAs of new *O*-methyltransferases (OMT), *CrOMT2* and *CrOMT4*, were isolated from *C. roseus* cell suspension cultures (grown in the dark) and were overexpressed in *E. coli*. The enzyme CrOMT4 was inactive with all substrates tested, whilst CrOMT2 was identified as a flavonoid OMT. It performs two sequential methylations at the 3'- and 5'-positions of the B-ring in myricetin (flavonol) and dihydromyricetin (dihydroflavonol), which is characteristic for *C. roseus* flavonol glycosides and

anthocyanins (Cacace *et al*., 2003). Schröder *et al*. (2004) used a homology based RT-PCR strategy to search for cDNAs encoding OMTs. They characterized a B-ring 4'OMT, CrOMT6, though 3',4'-dimethylated flavonoids had not been found so far in *C. roseus*. They also suggested that B-ring 3'-methylation is no hindrance for dioxygenases (such as flavanone 3β-hydroxylase, flavone synthase, flavonol synthase and anthocyanidin synthase) in flavonoid biosynthesis.

3.4 Regulation

3.4.1 Regulation of ICS, SA- and alkaloids production

In *C. roseus*, a fungal elicitor induced ICS activity (Poulsen *et al*., 1991; Moreno *et al*., 1994a). The ICS product is also a precursor of naphtoquinones (reviewed by Verberne *et al*., 1999). A hormone such as methyl jasmonate (MeJA) induces the ICS activity for stimulating anthraquinones (AQ) synthesis in *Galium mollugo* cell suspension cultures. ICS affinity for chorismate is lower than of other chorismate utilizing enzymes such as CM and AS preventing a large flux of substrate into the isochorismate pathway (Leduc *et al*., 1997). The regulation of ICS activity is also part of the regulation of AQ production in *Morinda citrifolia* (Stalman *et al*., 2003). The ICS activity is inhibited by auxins such as NAA and 2,4-D. ICS regulation can be different in different species. For example, in *Morinda citrifolia* the ICS activity and AQ production were reduced when the chorismate pool decreased by blocking the sixth metabolic step of the shikimate pathway (5-enolpyruvylshikimate 3-phosphate synthase, EC 2.5.1.19) by the herbicide glyphosate, whilst the opposite situation occurred in *Rubia tinctorum* cells (Stalman *et al*., 2003).

In *C. roseus*, different cell cultures showed different activation or inhibition pattern for enzymes upon elicitation. Seitz *et al*. (1989) showed that besides the induction of the alkaloid pathway, addition of a *Pythium* filtrate to a cell line of *C. roseus* cv. Little Delicata induced PAL activity and accumulation of phenolic compounds. Whilst, Moreno *et al*. (1994a) found that an increased activity of ICS paralleled the accumulation of 2,3-DHBA after elicitation of *C. roseus* A12A2 line with *Pythium aphanidermatum* extract. Effects of elicitation on different metabolic pathways in this *C. roseus* cell line were further observed (Moreno *et al*., 1996). AS and TDC were induced, resulting in an increased tryptamine level in the cells. CM was not induced, PAL activity was strongly inhibited but 2,3-DHBA accumulated in

the culture medium, indicating that another pathway than the phenylalanine pathway is involved for the production of this phenolic in *C. roseus* upon elicitation. Different amounts of *Pythium* extract and/or different enzyme analysis methods used, might also explain the different findings. A small amount of *Pythium* extract (0.5 - 2.5 mL) induced PAL activity but more than 2.5 mL provided reversed effects as determined by HPLC-measurement of *trans*-cinnamic acid, the direct product of PAL (Moreno, 1995).

In our experiments for selection for high-SA producing cell lines, the *C. roseus* A12A2 line (grown in M&S medium without growth hormones) showed the highest total SA after fungal elicitation. The *C. roseus* A11 line, grown in Gamborg B5 medium supplemented with NAA, produced a moderate level of total SA, whereas the lowest total SA was found in the CRPM line which was grown in M&S medium containing a combination of NAA and kinetin (10:1) (Chapter 4 of this thesis). Auxins (Woeste *et al*., 1999) and cytokinins (Cary *et al*., 1995) are known to induce ethylene synthesis in plants (e.g. *Arabidopsis* seedlings), but SA inhibits ethylene biosynthesis (Leslie and Romani, 1986). Auxin may act antagonistically with SA (Friedmann *et al*., 2003). Ethylene and jasmonate (JA)/methyl jasmonate (MeJA) are signaling compounds for induced systemic resistance (ISR) (van Wees *et al*., 2000). Thus, the presence of growth hormones in the medium might affect the CRPM cells to generate ISR rather than SA-dependent SAR. Plants generate either SA-dependent SAR or ISR depending on the plant species, the kind of elicitors (e.g. different pathogens), wounding, kind of herbivore, abiotic stress such as UV-light, drought, salinity and stress nutrients. In general, ISR works independently from SA-dependent SAR. However, a cross talk between the SA-dependent pathways and SA-independent pathways can occur in an attacked plant (van Wees *et al*., 2000; Pieterse *et al*., 2001; Kunkel and Brooks 2002). Some genetic studies with *Arabidopsis* reveal that the JA-dependent pathway can inhibit the SA-dependent pathway, and *vice versa*. Other studies show that either SA or JA can induce certain genes involved in SAR. Some ISR expressed genes require JA and ethylene, whilst the others only JA (reviewed by Glazebrook *et al*., 2003). Cross talk among these pathways can occur for a fine-tuning in SAR (Shah, 2003). Terpenoid indole alkaloids (TIAs) production in *C. roseus* is induced by MeJA (van der Fits and Memelink, 2000) but auxins were found to suppress the transcription of *TDC* and *STR* (some JA-responsive genes in TIA pathway). Whilst, addition of SA (0.1 mM) provided weak inducing effects on the

steady state of those mRNAs 8-24 h after treatment (Pasquali *et al*., 1992). Large increases in the specific content of TIAs and phenolic compounds were observed in media with high sucrose levels but lacking 2,4-D and some minerals (Knobloch and Berlin, 1981).

In an experiment using the *C. roseus* A12A2 cell suspension cultures fed with loganin and tryptamine, MeJA caused a high level of accumulation of strictosidine and ajmalicine, but SA decreased the level of ajmalicine compared to the control fed sample (El Sayed and Verpoorte, 2002). This might be a result of inhibition of the JA-dependent pathway by the SA-dependent pathway. However, an increase in enzyme activities or the transcription of a/some JA-responsive gene(s) in elicited plant cells may not be seen as activation of the JA-dependent pathway (ISR) only. A cross talk between JA- and SA-dependent pathways for fine-tuning SAR could happen for example in *C. roseus* A12A2 cell suspension cultures elicited by *Pythium* extract. The elicitation increased the ICS activity and the levels of SA and 2,3-DHBA (Budi Muljono *et al*., 2002), but induced also AS and tryptophan decarboxylase (TDC, EC 4.1.1.28) activities, and led to the accumulation of tryptamine (Moreno *et al*., 1996). However, strictosidine synthase (STR, EC 4.3.3.2) activity was not significantly induced and two enzymes from the TIA pathway: isopentenyl diphosphate isomerase (IPP-isomerase) and geraniol 10-hydroxylase (G10H) were inhibited. The alkaloid ajmalicine was not increased compared with the non-elicited (control) cells, showing the limitation of TIA(s) biosynthesis by blocking the activities of some other JA-responsive genes. *TDC* is regulated by *ORCA3* (Octadecanoid-Responsive Catharanthus AP2/ERF-domain) gene, which is induced by MeJA and elicitors (van der Fits and Memelink, 2000). In *C. roseus* A12A2 cells, *TDC* expression seems not inversely related to *ICS* expression and biosynthesis of SA upon elicitation with *Pythium*.

In some studies with *C. roseus* cell suspension cultures, auxins suppress not only *TDC*- but also *STR* expression, the level of alkaloids, the ICS activity and the level of 2,3-DHBA after *Pythium* elicitation as mentioned previously. Also, combination of auxin (NAA) and cytokinin (kinetin) strongly suppresses the SA level in *C. roseus* cell suspension culture (CRPM line). Interestingly, the combination of cytokinin and ethylene strongly enhanced the expression of *G10H* and clearly increased the expression of the MEP pathway genes (*DXS*, *DXR* and *MECS*) but did no effect *HMGR* (belonging to the mevalonate pathway), *TDC* and *STR* expressions in *C.*

roseus suspension cultures of C20D line. The hormones had no or little effect on the expression of these genes when they were given separately (Papon *et al*., 2005). The same *C. roseus* cell line showed a decrease in ethylene production when treated with cytokinin (Yahia *et al*., 1998). Combination of cytokinin-ethylene or cytokinin-auxin clearly shows different regulations for different parts of a TIA pathway. Apparently different signaling compounds can be employed and cross-talk among them can occur in the regulation of the secondary metabolite biosynthetic pathways. As discussed before, auxins also inhibited the ICS activity in *Morinda citrifolia* (Stalman *et al*., 2003) and ICS was induced by MeJA in *Galium mollugo* (Leduc *et al*., 1997) for accumulation of AQ. In *C. roseus*, increased levels of ICS activity paralleled the accumulation of 2,3-DHBA and SA upon a fungal elicitation. The presence of the ICS pathway leading to SA and whether the ICS gene is a JA-responsive gene requires further study. Figure 3.2 summarizes the effects reported for various plant hormones and signal compounds in *C. roseus* cell cultures.

In *C. roseus* seedlings, El Sayed and Verpoorte (2004) showed that MeJA was a general inducer for all alkaloids, but SA application increased also the production of serpentine and tabersonine, moreover it provided the highest level of vindoline compared to other hormone treatments. Auxins cause different effects in seedlings and suspension cell cultures, as a transient increase of TDC activity was found only in *C. roseus* seedlings (Aerts *et al*., 1992).

Sudheer and Rao (1998) reported that C6C1 compounds such as gentisic acid and 3,4-dihydroxybenzaldehyde enhanced the growth and total alkaloid content, but *p*-HBA provided opposite effects in *C. roseus* plants.

Since SA is important for signaling in SAR, cross talk between the shikimate- and phenylalanine pathway is possible. PAL up-regulation may not affect the isochorismate pathway, since ICS is not inhibited by aromatic amino acids (van Tegelen *et al*., 1999). The shikimate pathway exists in plastids (Herrmann and Weaver, 1999) and the phenylalanine SA pathway is thought to be present in the cytosol. Metabolic transport is clearly an important factor in regulation of SA synthesis. For example, SA can be synthesized in the plastids via the ICS pathway and subsequently exported to the cytosol, or synthesized from phenylalanine in the cytosol. The presence of small amounts of SA in tobacco plants overexpressing the genes encoding the bacterial pathway for SA without plastidial signal sequence can

also indicate the presence of a cytosolic pathway, which requires transport of chorismate/isochorismate out of the plastids (Verberne *et al*., 2000).

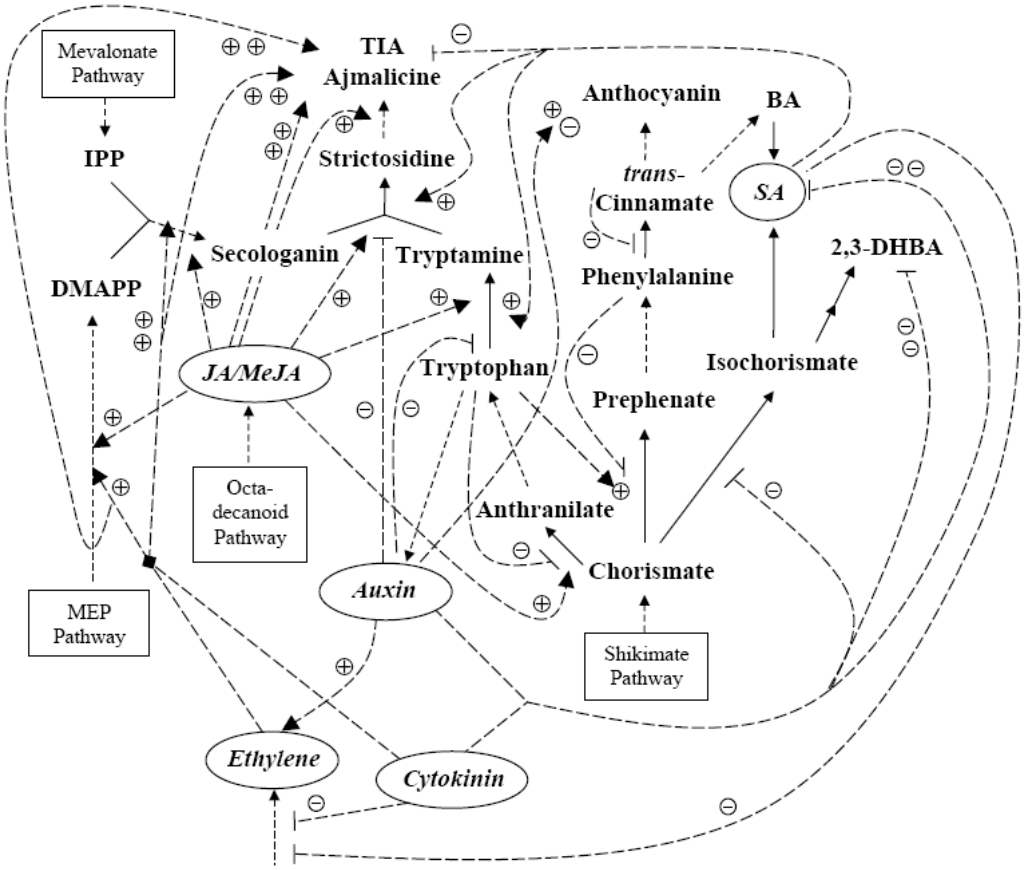

Figure 3.2. Summary of effects reported for various plant hormones and signal compounds in *Catharanthus roseus* cell cultures. A continued line means one-step reaction. A small-dashed line means multi-step reactions. A big-dashed line with + or - indicates activation or inhibition of gene(s) expression, enzyme activity or end product level. A big-dashed line with both + and - means a concentration-dependent activation or inhibition. A strong activation or -inhibition is indicated by ++ or - -.

3.4.2 Regulation of PAL, phenylpropanoids- and alkaloids production

Moreno *et al*. (1994b) showed that UV treatment of a *C. roseus* cell suspension culture (A12A2 line) stopped the cell growth and increased PAL activity. Addition of 2,3-DHBA into the cell cultures induced AS, STR and slightly TDC, whilst combined treatment with UV and 2,3-DHBA, strongly induced PAL-, AS-, STR-, TDC-activity, tryptamine accumulation and inhibited growth and G10H activity. As mentioned previously, elicitation with *Pythium* extract on this cell line strongly inhibited PAL activity (Moreno *et al*., 1996), showing the different gene regulation caused by different biotic/abiotic stresses.

PAL activity increased from 4 to 34 μkat/kg protein when a *C. roseus* cell culture was exposed to 1 mM 2,2'-azobis(2-amidinopropane)-dihydrochloride (=AAPH, a free radical-generating substance) (Ohlsson *et al*., 1995). The cells were grown in light on a half strength Gamborg B5 medium containing 2 mg/L NAA, 0.05 mg/L kinetin and 3% sucrose. Two days after an application of 5 mM AAPH, an increase of the content of phenolic substances in the medium (from 18 to 67 mg/mL, determined with chlorogenic acid as reference) was found. It is known, that generation of free radicals in plant cells, known as oxidative burst is part of the hypersensitive reaction (HR) as an early step before the onset of SAR (Ryals *et al*., 1996). Thus, exposing a plant to a free radical-generating substance can lead to SAR including PAL activation.

A study performed by Xu and Dong (2005) demonstrated that O_2^- rather than H_2O_2 was found to trigger PAL activation and catharanthine synthesis in *C. roseus* cell cultures. The cell culture was grown in a liquid M&S medium supplemented with 2 mg/L NAA, 2 mg/L IAA, 0.1 mg/L kinetin and 3% sucrose in the dark. O_2^- generated by the reaction of xanthine/xanthine oxidase, without the presence of elicitor (*Aspergillus niger* cell wall components), was able to activate PAL and catharanthine synthesis and to reverse the inhibitory effect of diphenylene iodonium (DPI) on elicitor-induced PAL activation and catharanthine synthesis. External application of H_2O_2 and catalase had no effect on those plant defense responses.

The study discussed above shows the activation of PAL and the production of alkaloids upon an abiotic stress in the presence of growth hormones. Another study revealed that competition for the carbon source may occur between the phenylpropanoid pathway and TIA pathway. For example, elicitation of *C. roseus* cell suspension culture by biotic stress (a fungal elicitor) in the presence of *trans*-cinnamic acid (a PAL inhibitor) increased the alkaloid production (300% higher than non-

treated cells) 72-h after treatment (Godoy-Hernandez and Loyola-Vargas, 1991). Scaling up a *C. roseus* cell suspension culture from 250 mL to a 14-L bioreactor decreased the total alkaloid production more than 80%. But combination of osmotic stress and the inhibition of PAL activity by adding 1 mM *trans*-cinnamic acid into the bioreactor restored the original alkaloid amounts (Godoy-Hernandez *et al*., 2000). Caffeic acid and ferulic acid were found to enhance the growth and total alkaloid content in *C. roseus* plants, whereas *p*-coumaric acid showed opposite effects (Sudheer and Rao 1998).

3.4.3 Regulation of C6C3C6 and alkaloid biosynthesis

Light induces the production of some anthocyanins detected as anthocyanidins (malvidin, petunidin) in a callus culture of *C. roseus* 21 days after inoculation (Carew and Krueger, 1976). The callus culture originated from a *C. roseus* callus grown in the dark and which was transferred in a Gamborg agar medium (PRL 1), subcultured and then placed under 2150 lux continuous cool ray fluorescent light. Increasing light intensity and by adding a precursor like either phenylalanine or *trans*-cinnamic acid (100 mg/L) into the medium, increased the accumulation of the pigments. Removal of the light source inhibited pigment accumulation and increasing the sucrose concentration (2%) also decreased the accumulation.

Knobloch *et al*. (1982) found the same anthocyanidins in medium-induced cell suspension cultures of *C. roseus*. This group studied the influence of environmental factors such as medium composition and light on the accumulation of ajmalicine, serpentine, phenolics, and anthocyanins as well as on the growth rate of the cells. Transferring a 2-week-old cell suspension culture (grown in M&S medium with 2 μM 2,4-D in the dark) into a 10-fold volume of an 8% aqueous sucrose solution in the dark, caused accumulation of ajmalicine, but no anthocyanins were detected after 2 weeks incubation. Continuous illumination of this medium-induced suspension cells leads to a lower level of ajmalicine but a considerable amount of the oxidation product of ajmalicine (serpentine), an increased level of phenolics and the accumulation of anthocyanins. Interestingly, only about 5% of the cells in a culture showed a high content of anthocyanins (red color). Hall and Yeoman (1986) reported that anthocyanin production in *C. roseus* cell cultures is determined by the percentage of producing cells. The accumulation levels in all the producing cells are very similar, pointing to a feedback inhibition mechanism controlling the anthocyanin

concentration. The percentage of producing cells never exceeded 20%. A similar situation was found by microscopic analysis for the serpentine-producing cells. The optimal effect of light to stimulate the formation of anthocyanins and serpentine required low concentrations of 2,4-D, phosphate and mineral nitrogen (Knobloch *et al*., 1982). Quercetin was found to inhibit the growth and total alkaloid content in *C. roseus* plants (Sudheer and Rao, 1998).

3.5 Conclusion

Either biotic or abiotic stress or a combination of both increases the production of phenolic compounds in *C. roseus*. Different kinds of stress may affect different parts of the SAR pathways and may determine whether SA, JA, ethylene or more than one signaling compound is employed in a plant species such as in *C. roseus*. A cross talk between the SA-dependent- and the SA-independent pathways may result in induction of different pathways for the production of phenolic compounds and/or other secondary metabolites. For example, biosynthesis of SA can employ either the ICS pathway or the phenylalanine pathway, which may depend on many factors including the kind of stress. This may result in e.g. activation of a part of the TIA pathway and inhibition of other parts. The results of the SAR studies in other plant species can give important information for a comparison, but one should be careful not to generalize those, because many factors determine the activation or inhibition of a pathway even within a species. The defense responses can be different for different cultivars or for intact plants, seedlings, plant cell cultures, or even cell types.

Unraveling the biosynthetic pathway of phenolic compounds like SA upon stress in *C. roseus* will be useful to develop strategies for increasing alkaloid production by engineering metabolic pathways in this plant. If the isochorismate pathway is responsible for the synthesis of SA necessary for SAR in the cells (as in the case of 2,3-DHBA), it is interesting to know why the induction of the ICS activity parallels the induction of TDC, which is a product of a JA-responsive gene. Elicitation with *Pythium* may activate both JA- and SA- regulated genes or possibly *ICS* is also a JA-responsive gene, as in *Galium mollugo* cells ICS is induced by MeJA in connection with the accumulation of AQ. Combinations of growth hormones such as cytokinin-ethylene activates some genes from the terpenoid pathway and the MEP pathway resulting in increased levels of ajmalicine, but had no effect on *TDC* and *STR* expression. These results are in accordance with the finding that the terpenoid

pathway is a limiting factor for alkaloid biosynthesis. Upon fungal elicitation, the activities of TDC and STR increased in parallel with the biosynthesis of SA. The SA pathway after elicitation is strongly suppressed by a combination of cytokinin-auxin.

From the various studies it is clear that the different secondary metabolites pathways are part of a complex network that is regulated by a combination of factors, including some signal compounds. For example, activation of PAL and alkaloid biosynthesis needs further investigation as competition for the carbon source between phenylpropanoid pathway and TIA pathway may occur. A better insight in the regulation of the various secondary metabolite pathways in *C. roseus* will thus be important. The combination of genomic, transcriptomic, proteomic and metabolomic approaches will be an important tool for unraveling the SAR controlled-pathways including the biosynthetic pathways of the desired valuable secondary metabolites.

Chapter 4

Salicylic acid production in *Catharanthus roseus* cell suspension cultures elicited by *Pythium aphanidermatum* extract

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Abstract

The production of salicylic acid (SA) and salicylic acid glucoside (SAG) in *Catharanthus roseus* (L.) G.Don cell cultures increased after elicitation with a *Pythium aphanidermatum* (Edson) Fitzpatrick CBS 313.33 extract. Among five cell lines assayed, A12A2, which is grown in a 2% glucose containing Murashige & Skoog liquid medium without growth hormone, produced the highest amount of total salicylic acid (about 3.5 μg/g fresh weight) 24 h after elicitation.

Keywords: salicylic acid, *Catharanthus roseus* cell suspension cultures, *Pythium aphanidermatum*

4.1 Introduction

Salicylic acid is a natural product, which was developed from salicin extracted from willow bark and leaves. It was first successfully used in 1874 for the therapy of rheumatism. Now, the acetyl derivative of this phenolic compound, Aspirin, is widely used not only as a non-steroid anti-inflammatory drug (NSAID) but also as an anticlotting agent to prevent heart attack (Vane and Botting, 2003).

In plants, this compound plays an essential role in several physiological processes such as flowering, ion uptake, stomatal closure, heat production, and disease

resistance (reviewed by Raskin, 1992). Since it was reported that SA induced resistance to viral infections in tobacco leaves (White, 1979), the interest to understand the role of this phenolic acid in the systemic acquired resistance (SAR) increased. This led to many studies done in this field in order to elucidate the SAR pathway, including the biosynthetic pathway of SA as a part of the whole SAR response.

Two different biosynthetic pathways leading to SA (reviewed by Verberne *et al*., 1999) have been reported up to now (Figure 4.1). One pathway is via phenylalanine and cinnamic acid. Chorismate mutase (CM, EC 5.4.99.5) using the substrate chorismate, is the first enzyme in this so-called phenylpropanoid pathway. Deamination of phenylalanine catalyzed by the well-known enzyme phenylalanine ammonia-lyase (PAL) leads to cinnamic acid, the precursor of SA. This pathway has been suggested to be present in plants. Another pathway employs isochorismate synthase (ICS, EC 5.4.99.6) converting chorismate into isochorismate, and isochorismate pyruvate-lyase to convert isochorismate into SA. In microorganisms, SA, as well as 2,3-dihydroxybenzoic acid (2,3-DHBA) are precursors for siderophores formation (e.g. enterobactin, pyochelin) for uptake of Fe^{3+} in an aerobic growth condition where this ion is highly insoluble. This Fe^{3+} uptake is not only essential for the survival of microorganisms, but also in the virulence of several bacteria like *Escherichia coli, Bacillus subtilis, Mycobacterium smegmatis* and *Pseudomonas aeruginosa* as reported by Weinberg (1978).

The complete phenylpropanoid pathway towards SA has still not been resolved (reviewed by Verberne *et al*. 1999), however the presence of the isochorismate pathway leading to SA in *Arabidopsis thaliana* has been indirectly proved by the cloning of the genes (Wildermuth *et al*., 2001). A retrobiosynthetic study of 2,3-DHBA formation in *Catharanthus roseus* cell suspension cultures has shown the existence of the isochorismate pathway leading to 2,3-DHBA in this plant (Budi Muljono *et al*., 2002).

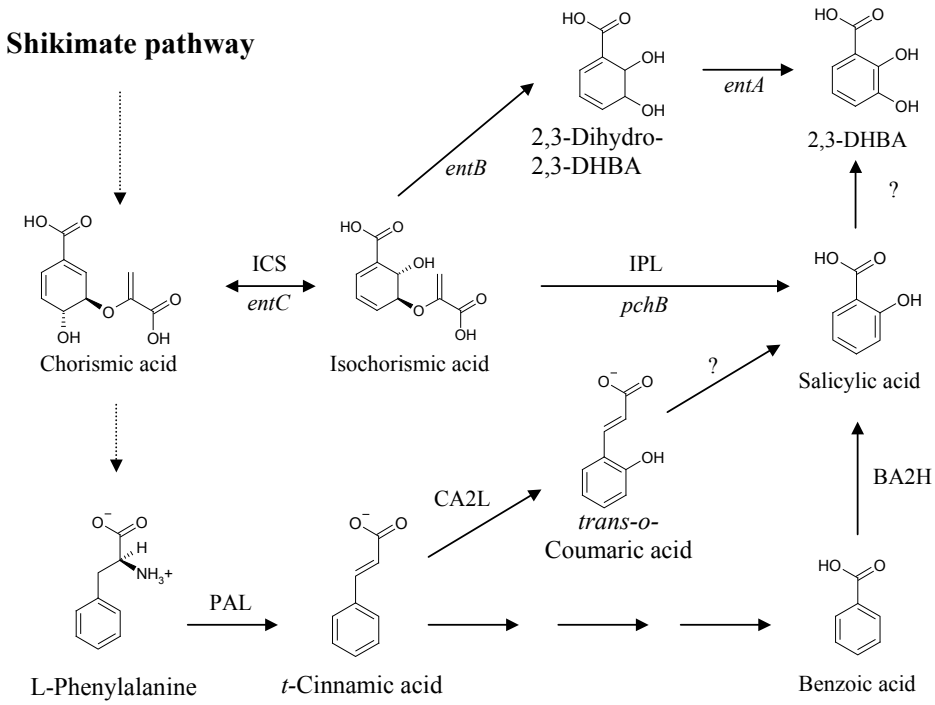

Figure 4.1. Biosynthetic pathway and the enzymes or genes involved in the biosynthesis of salicylic acid and 2,3-dihydroxybenzoic acid in plants via the phenylpropanoid pathway and in micro-organisms via the chorismate/ isochorismate pathway (Verberne, 2000). DHBA = dihydroxybenzoic acid; BA2H = benzoic acid 2-hydroxylase; CA2L = coumaric acid lyase; ICS = isochorismate synthase; PAL = phenylalanine ammonia lyase; *ent* = *E.coli* gene; *pchB* = salicylate synthase gene of *Pseudomonas aeruginosa*.

Since SA is normally produced by most plants in trace amounts only, it is necessary to find a suitable model that is able to provide an appropriate amount of SA for the labeled precursor feeding experiments, using NMR analysis for establishing the sites of the labels. The *C. roseus* cell cultures could be a suitable system allowing the comparison with 2,3-DHBA as control for the incorporation of the label. To increase the SA production level in *C. roseus* cells, elicitation could be a solution. Moreno *et al*. (1994a) and Frankmann and Kauss (1994) reported the accumulation of 2,3-DHBA in *C. roseus* cell cultures after inducing the ICS activity with a fungal elicitor. Budi Muljono (2001) used various elicitors such as cellulase, pectinase, yeast extract and *Pythium aphanidermatum* extract for *C. roseus* cell suspension cultures to compare their effect on ICS activity and the level of phenolic compounds (SA and 2,3-DHBA). It was shown that the activity of ICS was increased in the fungal- and

yeast-treated cells, whereas only low levels of ICS activity were found in both cellulase- and pectinase-treated cells.

Here, the production of SA in five different lines of *C. roseus* suspension cultures elicited by *Pythium* extract is reported.

4.2 Material and methods

4.2.1 Plant cell cultures

Five different cell lines of *C. roseus* were grown in Murashige and Skoog (M&S) medium (Murashige and Skoog, 1962) or Gamborg B5 medium (Gamborg *et al*., 1968), which were supplemented with 2% of D(+)-monohydrate glucose as the carbon source. The five cell lines and the growth media are shown in Table 4.1. The cells were grown in 250 mL-Erlenmeyer flasks containing 100 mL medium and cultivated at 24 – 25 °C under continuous light (500-1500 lux), on a shaker at 100 rpm for 5 days prior to elicitation.

Table 4.1. The different cell lines of *Catharanthus roseus* used in the *Pythium aphanidermatum* elicitation experiments.

	Catharanthus roseus (L.) G.Don			*C. roseus* "Pacific punch"	*C. roseus* "Pauline" 19940132
Line's name:	CRPM	A11	A12A2	CRPP 93	CR Pauline
Medium:	M&S (58)	Gamborg B5	M&S (9)	Gamborg B5	M&S (9)
NAA:	2.0 mg/L	1.86 mg/L	-	1.86 mg/L	-
Kinetin:	0.2 mg/L	-	-	-	-
Glucose:	20.0 g/L	20.0 g/L	20.0 g/L	20.0 g/L	20.0 g/L
Subculturing time:	1 week	3 weeks	1 week	3 weeks	1 week
Cell color:					
Before elicitation:	creamy	dark green	creamy/ yellowish	apple green	apple green
24 h elicitation:	creamy	olive green	brownish	dark green	olive green
48 h elicitation:	creamy/a bit dark	dark olive-green	dark brown	dark green	brown/dark olive-green

4.2.2 Elicitor

Pythium aphanidermatum (Edson) Fitzpatrick CBS 313.33 was used as the elicitor. This fungus was maintained on malt extract agar medium at 25 °C, in the dark and subcultured every 4 weeks. Aseptically, the solid culture was cut in pieces

and two pieces (each about 1 cm^2) were transferred into a 250 mL-Erlenmeyer flask containing 100 mL M&S liquid medium with 3% sucrose. This culture was then cultivated at 27 °C on a shaker at 100 rpm for 7 days. It was then sterilized in an autoclave (120 °C, 20 min) and subsequently filtered under aseptic conditions. The filtrate was used as the elicitor.

4.2.3 Elicitation

Ten ml of the *Pythium* extract was added to 100 mL of 5 days old *C. roseus* – suspension cells. The cultivation conditions for the treated cultures were the same as that for culture maintenance. The elicited cells were harvested 24 h and 48 h after treatment by filtration using a P2 filter to separate the cells from medium. The cells were frozen in liquid nitrogen and used for SA and SAG analysis.

To study the effect of different amounts of *Pythium* extract on SA level in *C. roseus* cells, 10 mL, 20 mL or 30 mL of the extract were applied to 3 flasks containing 100 mL A11 suspension cells. The cells were harvested 24 h after the treatment and 2 samples were taken from each flask for SA analysis.

4.2.4 Chemicals

4.2.4.1 Chemicals used for the medium of cell suspension cultures

The chemicals used in Macro Murashige & Skoog (M&S) or –Gamborg B5 salts were: $CaCl_2$ (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 $MgSO_4$ exsiccatus BP was obtained from OPG Farma (BUVA BV, Uitgeest, The Netherlands). The chemicals used in Micro M&S or –Gamborg B5 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) were dissolved in one solution, whereas others such as KI, $NaMoO_4.2H_2O$, $CuSO_4.5H_2O$ and $CoCl_2.6H_2O$ (from Merck) were dissolved in another solution due to the problem of solubility. Thiamine-di-HCl was from Janssen Chimica (Geel, Belgium). Pyridoxine-HCl was from Sigma-Aldrich Chemie (Steinheim, Germany). Nicotinic acid (99.5%) and glycine (99.7%) were purchased from Merck. 1-Naphtaleneacetic acid (NAA) and kinetin were from Merck (Schuchardt, Germany). Sucrose (99.7%) and myo-inositol (>99.0%) were from

Duchefa Biochemie (Haarlem, The Netherlands). D(+)-Glucose (>99.0%) was obtained from Fluka Chemie (Buchs, Germany).

4.2.4.2 Chemicals used for determination of salicylic acid

Salicylic acid was obtained from Sigma (St. Louis, MO). Methanol (>99.8 %) and ethyl acetate (>99.8 %) were from Biosolve BV (Valkenswaard, The Netherlands). *n*-Hexane (>99 %), acetic acid (100 %) and hydrochloric acid (36-38 %) were purchased from Mallinckrodt Baker BV (Deventer, The Netherlands). Sodium acetate trihydrate (99.5-101.0 %) and trichloroacetic acid (>99.5 %) were from Merck (Darmstadt, Germany). Sodium hydroxide (> 99%) was from Boom (Meppel, The Netherlands).

4.2.5 Extraction

The cells were ground in the presence of liquid nitrogen, using a mortar and a pestle. This ground cell material (400 mg) was placed in a 2 ml-micro tube and extracted following the method described by Verberne *et al*. (2002). One ml of 100 % methanol was used for the first extraction. The mixture was vortexed (2,500 rpm, 1 min) using a Vibrofix VF1 electronic vortex (IKA, Staufen, Germany), followed by sonication (5 min) in an ultrasonic bath and centrifugation (13,000 rpm, 10 min) using a BHG HermLe Z 231 M centrifuge (B. HermLe, Gosheim, Germany). The supernatant was then collected. Extraction of the pellet was repeated using 0.5 mL of 100 % methanol, following the steps described in the previous extraction. This supernatant was combined with the first supernatant and 10 µl of 0.2 M sodium hydroxide was added. Evaporation of this water-methanol mixture was carried out in a Savant SpeedVac Plus SC110A concentrator (New Brunswick Scientific BV, Nijmegen, The Netherlands) at medium drying speed. The residue was then acidified again with 250 µl of 5 % (w/v) trichloroacetic acid (TCA) and vortexed before partitioning twice with 800 µl of ethyl acetate : *n*-hexane (1:1). The non-polar fractions were collected, 60 µl of HPLC eluent (0.1 M sodium acetate buffer pH 5.5 : methanol, 10:1 v/v) was added, and then evaporated to an adjusted-volume of 60 µl. This evaporation residue was diluted by addition of 600 µl of HPLC eluent and centrifuged before injection into HPLC for free SA analysis. The SAG remaining in the TCA fraction was hydrolyzed by addition of 300 µl of 8 M HCl and incubation at

80°C for 1 h. After the acid hydrolysis, the SA obtained was extracted by partitioning the residue with ethyl acetate : *n*-hexane (1:1 v/v) following the steps described as those for free SA before injection into HPLC.

4.2.6 Determination of salicylic acid recovery

Four samples of ground cells (each of about 400 mg fresh weight) were spiked with 0.5 μg SA and extracted following the method described in 4.2.5 for free SA analysis. Another four non-spiked samples from the same cell material with the same weight were extracted as well, and the TCA-water phases were then spiked with 0.5 μg SA before acid hydrolysis and subsequently extracted for the analysis of SA after hydrolysis. Another four non-spiked samples (the same weight and origin as the previous samples) were extracted for their endogenous free SA and –bound SA analysis. Four SA standard solutions of 0.5 μg SA in 660 μl HPLC buffer were also made. After centrifugation, all of the extracts (spiked and non-spiked) and the SA standard solutions were injected into HPLC.

The mean of the peak area differences between peak areas of spiked samples and those of non-spiked samples, was then corrected with the mean of peak areas of SA standard solutions to have the SA recovery and the bound-SA recovery. This experiment was performed in triplo.

4.2.7 Calibration curve

A calibration curve was made in every series of quantitative analyses. A stock solution of SA (1mg/mL) was diluted to get a concentration of 0.05 mg/mL. Subsequently, from this concentration, via dilution (in duplo), a range of concentrations was obtained and injected in the HPLC system. The mean of the resulting peak areas was used for the regression equation.

4.2.8 HPLC analysis

HPLC analysis of SA was performed based on the system described by Verberne *et al*. (2002). The column, a Phenomenex column type LUNA 3μ C18 (2) 150 x 4.60 mm equipped with a SecurityGuard from Phenomenex (Torrance, CA, USA) was used. The mobile phase consisted of 0.1 M sodium acetate buffer pH 5.5 : methanol (10:1 v/v) and was pumped using an LKB, 2150 HPLC pump (Bromma, Sweden), at

a flow rate of 0.80 ml/min. The injection (20 µl) of the samples was performed using a Gilson 234 auto-injector (Villiers Le Bel, France). The detection was carried out using a Shimadzu RF-10AxL spectrofluorometric detector (Tokyo, Japan), at an emission wavelength of 407 nm and an excitation wavelength of 305 nm, which was connected to a CR 501 Chromatopac printer (Shimadzu, Kyoto, Japan). The SA peak appeared at around 13-14 min.

4.3 Results and discussion

Plants normally produce salicylic acid in trace amounts. In order to study the SA biosynthetic pathway, a suitable plant model, which is able to produce a relatively high amount of SA for extraction and ^{1}H-NMR-analysis, is needed. Here, we used *C. roseus* cell suspension cultures, which previously showed to be a suitable model for a retrobiosynthetic study of 2,3-DHBA formation (Budi Muljono *et al*., 2002). For studying the SA production in different systems, a reliable analysis method is required. This method should take into account the relatively low concentration of this compound in the extract and its sublimation at relatively low temperature. The analysis method developed by Verberne *et al*. (2002) was chosen in this experiment.

The experiments for free SA- and SA after acid hydrolysis recoveries were carried out three times with the mean result of 85.79% (SD ± 7%) for the free SA recovery and 82.79% (SD ± 1%) for the recovery of SA after acid hydrolysis. For quantitative determination, free SA and bound SA of both control and elicited-cells of each cell line were measured in triplo. This experiment was performed at least twice for a cell line. The free SA and bound SA levels in five cell lines, which were obtained after correction with the recovery rate, are shown in Figure 4.2.

To elicit the plant cells, *Pythium aphanidermatum* was used in this experiment because this fungus has been proven to be an effective elicitor for *C. roseus* suspension cultures (Moreno *et al*., 1994a; Frankmann and Kauss, 1994; Budi Muljono, 2001). Budi Muljono (2001) proved that the accumulation pattern of 2,3-DHBA and SA correlated well with ICS activity. Using an analysis method different from that applied in our experiment, the highest levels of free SA and bound SA were found to be about 0.25 µg/g fresh weight (FW) and 0.19 µg/g FW 20 hr after treatment, in yeast-treated cells. Whilst, in the fungal-treated cells, the highest levels of free SA (about 0.33 µg/g FW) and bound SA (0.03 µg/g FW) were found 24 hr

after treatment (Budi Muljono, 2001). In our experiment, elicitation of all cell lines resulted in an increased level of free SA and bound SA compared to the controls (Figure 4.2).

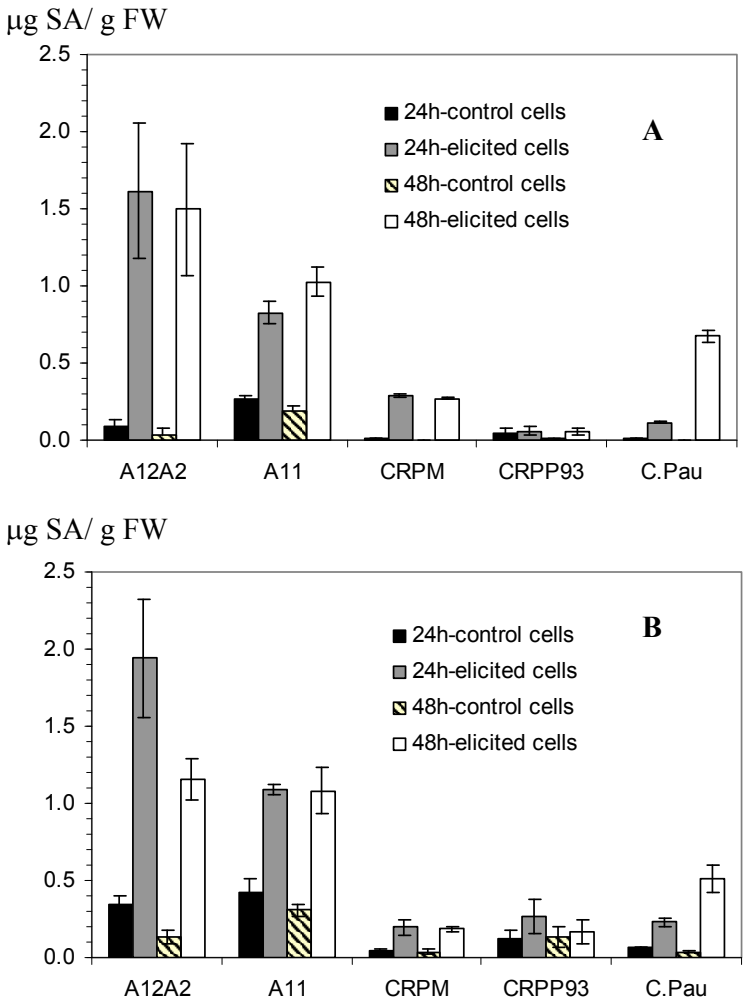

Figure 4.2. Free salicylic acid (A) - and SA after acid hydrolysis/SAG (B) levels in some cell lines of *Catharanthus roseus* cell suspension cultures after elicitation with 10 mL *Pythium aphanidermatum* extract. Determination of SA level in each line used more than one batches, where the independent experiments were performed at different days (*n* =3).

Among the five cell lines, 24 hr-elicited cells of *C. roseus* A12A2 produced the highest amount of total SA (free SA and bound SA) of 3.56 µg/g FW. Twenty four hours after elicitation, the A11 cell line color changed to olive green, and became darker 48 h after treatment, while the color of A12A2 changed to brownish (24 h) and dark brown. The oxidative burst, a phenomenon that is part of SAR and contributes to cell death (Ryals *et al*., 1996) shown by a dark brownish color, probably occurred

more intensively in A12A2 than in A11 cells. The only slight change in color found in CRPM cells may point to the absence of the oxidative burst. Considering the high levels of SA produced, both A12A2 and A11 cell lines could be used for mapping the SA pathway.

The effect of different amounts of *Pythium* extract on the SA-level in A11 was studied. After 24 h, the 20 ml of *Pythium* extract resulted in a slightly higher production of total SA in the cells as shown in Figure 4.3.

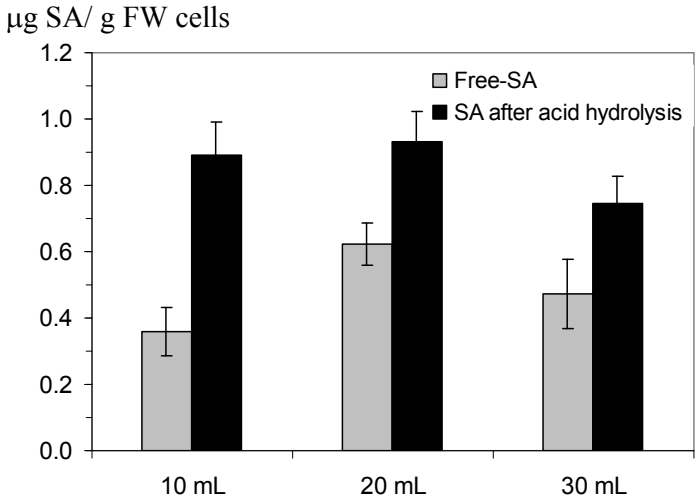

Figure 4.3. Effect of different amounts of *Pythium* extract on the level of salicylic acid in A11 cells, 24 hour after treatment. Three batches were used for each treatment and the SA analysis of each batch was performed in duplo.

A12A2 was originally derived from the same variety as A11 and CRPM, *C. roseus* (L.) G.Don, but differed in the nutritional treatment (Table 4.1). The absence of growth hormone in the medium caused probably the highest production of SA in A12A2 cells 24 h after elicitation. A11 that was fed with medium containing a synthetic auxin 1-naphtaleneacetic acid (NAA), produced a lower amount of SA than A12A2 did. The presence of a combination of NAA and kinetin (a synthetic cytokinin) in the medium may be the cause of the low level of SA in CRPM cells compared with A12A2 and A11. Stalman *et al*. (2003) studied the effects of growth hormones on the secondary metabolite production in plant cell cultures. It was shown that *Morinda citrifolia* cell culture grown on a medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), a potent synthetic auxin, were very stable with virtually no production of anthraquinones (AQ). The presence of NAA in the medium

provided a relatively stable culture and a moderate amount of AQ (15 μmol/g FW), whereas a cell culture that was grown without any growth hormones was unstable and produced a high amount of AQ (40 μmol/g FW), which subsequently led to cell death. This group also showed that biosynthesis of AQ in *Morinda* cells were modulated by ICS activity. The auxins 2,4-D and NAA both repressed AQ formation and ICS in a correlated fashion, of which 2,4-D had about 30 times more inhibitory effect than NAA. In *C. roseus* cell cultures, the production of SA and 2,3-DHBA increased after elicitation with *Pythium* extract and was preceded by an increase of ICS activity (Budi Muljono, 2001). Though we have not investigated the correlation between the effect of growth hormones and ICS activity upon elicitation with *Pythium* extract, the information discussed above provides a clue that ICS activity could correlate with the repressed level of SA caused by the growth hormones. The low level of SA in elicited CRPM cells may indicate that the system generates an SA-independent defense response, employing other signaling compounds such as ethylene or jasmonate (JA). Exogenous application of auxins is known to induce the synthesis of ethylene (Woeste *et al*., 1999). Ethylene signaling is required in addition to JA signaling for the expression of some genes involved in induced systemic resistance, also several lines of genetic evidence show that SA signaling can inhibit JA signaling (Glazebrook *et al*., 2003). Another example showing the opposite effects of SA and ethylene in plant physiological processes is for example in the gravitopic bending process of flowering shoots (Friedmann *et al*., 2003).

4.4 Conclusion

Elicitation of *Catharanthus roseus* cell suspension cultures with *Pythium aphanidermatum* extract increased the level of total SA in the cells. The *C. roseus* A12A2 cell line (grown in a hormone-free Murashige & Skoog medium) provided the highest level of total SA (free SA and bound SA) 24 h after treatment with 10 mL *Pythium* extract. The optimum amount of *Pythium* extract to produce the highest level of SA in the *C. roseus* A11 cell line was 20 mL. *Catharanthus roseus* A12A2 and A11 cell lines can be used for future SA retrobiosynthetic studies.

Chapter 5

Single step purification of salicylic acid from *Catharanthus roseus* cell culture (plant material) by anion exchange for NMR analysis

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Abstract

Salicylic acid (SA) is an important plant-signaling compound for inducing systemic acquired resistance. A simple single step ion exchange chromatography method has been developed for purification of SA from plant cell culture extracts to obtain a relatively clean extract for NMR analysis, as a tool to measure ^{13}C-isotope incorporation pattern in retrobiosynthetic studies of SA. By measuring ^{13}C-incorporation in SA it will be possible to determine the biosynthetic pathway(s) involved as well as measuring the flux through the pathways. A system containing 2 g Dowex 1WX2 (100 mesh) as the resin, 25 mM sodium phosphate pH 7.0-7.5 as the washing solvent and 0.3 M HCl in 60% acetonitrile as the elution solvent provides the most efficient system for an elution of 50 μg SA standard. The retention volume of SA changes depending on the ratio of the amounts of SA in the extract/resin. The SA recoveries are 69 – 80% (in total first 3 x 10 mL elution) calculated from the whole purification process. Depending on the amount of SA in an extract, the amount of the resin can be determined to obtain the best elution profile with most of SA concentrated in one fraction. Seventy μg SA/g resin yielded 65% SA recovery in the second 10 mL elution (184 μg SA), which provided a well-detectable amount of SA with a relatively low level of impurities as analyzed by 400 MHz ^{1}H-NMR.

Keywords: salicylic acid, plant cell cultures, ion exchange chromatography, ^{1}H-NMR

5.1 Introduction

SA has several physiological functions in plants (Raskin, 1992) and serves as a precursor for chelating agents in bacteria (Weinberg, 1978). One of the important functions of SA in plants is to induce systemic acquired resistance (SAR) generated by biotic or abiotic stress. SAR is characterized by increased levels of some acidic pathogenesis related (PR) proteins and phytoalexins, which have anti-microbial activities (Ryals *et al*., 1996). SA is a C6C1 compound that is synthesized from chorismate, the end product of the shikimate pathway and a substrate for 5 enzymes leading to a diversity of secondary metabolites in plants (reviewed by Mustafa and Verpoorte, 2005). By chorismate mutase (EC 5.4.99.5), chorismate is transformed into prephenate and subsequently (by some steps) into phenylalanine. SA in plants is thought to be derived from phenylalanine, whereas microorganisms employed the enzymes isochorismate synthase (EC 5.4.99.6) and isochorismate pyruvate-lyase (IPL) to transform chorismate into isochorismate and then directly into SA (reviewed by Verberne *et al*., 1999). However, it might also be possible that the isochorismate pathway leads to SA in plants (Verberne *et al*., 2000), which was indirectly proven in *Arabidopsis* (Wildermuth *et al*., 2001). Under normal conditions, plants produce SA only in trace amounts, but the level of SA rapidly increases upon pathogenic attack by e.g. tobacco mosaic virus in tobacco (Verberne *et al*., 2000). In *Catharanthus roseus* (L.) G.Don cell cultures, elicitation causes the induction of SA biosynthesis (Budi Muljono, 2001) and 2,3-dihydroxybenzoic acid via the isochorismate pathway (Budi Muljono *et al*., 2002).

The trace amount of SA in plants has made it difficult to find a suitable method for analysis of this compound from plants. Up to date either gas chromatography (GC) or high performance liquid chromatography (HPLC) has been developed for the analysis of SA from plants (Budi Muljono *et al*., 1998; Raskin *et al*., 1989; Verberne *et al*., 2002). Among the previous methods an HPLC separation combined with fluorescence detection offers the optimum for the quantitative SA analysis in terms of selectivity (detects fluorescence compounds only) and sensitivity (in nanograms level). However, this method is not satisfactory for biosynthesis studies on SA. Such studies require the isolation of sufficient SA for NMR spectrometric measurements to determine incorporation positions of e.g. ^{13}C-labels, similar as has been done for 2,3-dihydroxybenzoic acid (Budi Muljono *et al*., 2002). Such studies will also allow

analysis of carbon fluxes through the pathway(s) involved. The goal is thus to develop a simple purification method of SA from a large amount of cells that gives a sample, in which the SA signals can be observed in the NMR without overlap of other compounds.

In developing a purification method, the physicochemical properties of the target compound should be taken into account. SA belongs to the group of phenolic acids. This structure allows the compound to be separated from an aqueous extract under acidic conditions by partitioning with a non-polar solvent or purification by ion exchange chromatography (IEC). For example IEC was used to extract benzoic acid from landfill leachate, a liquid formed by the degradation of the organic matters present in landfill, which can contaminate the soil, superficial- and ground water (Nascimento Filho *et al*., 2004). Also, anion exchange chromatography was employed to further clean up an aliquot fraction obtained from pre-purification of plant extracts using polyamide column chromatography (Klick and Herrmann, 1988). These purification steps were part of the procedure developed for a GC / HPLC analysis of a series of phenolic acid glucosides such as 4-β-D-glucosides of vanillic-, syringic-, 4-hydroxybenzoic-, protocatechuic- and gallic acid, salicylic acid 2-*O*-β-D-glucoside and the 1-*O*-β-D-glucose esters of 4-hydroxybenzoic-, vanillic- and syringic acid from plant extracts.

Considering the property of SA as an acid with a moderately low pKa of 3.0 (Hanai *et al*., 1999), we chose IEC using a strong anion exchanger Dowex 1WX2 (100 mesh) as the resin for purification of this compound from plant cell culture extract to obtain a relatively clean extract for NMR analysis as a tool in a retrobiosynthetic study of SA. In the present study, we developed and optimized an IEC system using pure SA and subsequently applied this method to plant extracts. The aim of this purification step is to obtain a relatively pure- and well-detectable amount of SA in the fraction for analysis by ^{1}H-NMR spectrometry.

5.2 Materials and methods

5.2.1 Chemicals

5.2.1.1 Chemicals used for the medium of cell suspension cultures

The chemicals used in Macro Murashige and Skoog/M&S salts (Murashige and Skoog, 1962) or Macro Gamborg B5 salts (Gamborg *et al*., 1968): $CaCl_2$ (min. 99%), KH_2PO_4 (min. 99.5%), KNO_3 (min. 99%) and NH_4NO_3 (min. 99%) were obtained from Merck (Darmstadt, Germany) and $MgSO_4$ exsiccatus BP was purchased from OPG Farma, BUVA BV (Uitgeest, The Netherlands). The chemicals used in Micro M&S or Micro Gamborg B5 salts: $FeSO_4.7H_2O$ (Brocades-ACF groothandel NV, Maarssen, The Netherlands) and H_3BO_3, $MnSO_4.H_2O$, Na_2EDTA, $ZnSO_4.7H_2O$ (Merck) were dissolved in one solution, whereas others including $CoCl_2.6H_2O$, $CuSO_4.5H_2O$, KI, $NaMoO_4.2H_2O$ (Merck) were dissolved in another solution due to the problem of solubility. Glycine (99.7%), 1-naphthaleneacetic acid (NAA), and nicotinic acid (99.5%) were from Merck (Schuchardt, Germany). D(+)-glucose (> 99.0%) was obtained from Fluka Chemie (Buchs, Germany). myo-Inositol (> 99.0%) and sucrose (99.7%) were from Duchefa Biochemie (Haarlem, The Netherlands). Pyridoxine-HCl was from Sigma-Aldrich Chemie (Steinheim, Germany). Thiamine-*di*-HCl was from Janssen Chimica (Geel, Belgium).

5.2.1.2 Chemicals used for extraction, separation and analysis of salicylic acid

Dowex 1WX2 100 mesh (replacement for 1X2100) and salicylic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (MeOH, > 99.8%), acetonitrile (AcCN, > 99.8%) and ethyl acetate (EtOAc, > 99.8%) were from Biosolve BV (Valkenswaard, The Netherlands). Cyclohexane (> 99%), acetic acid (100%) and hydrochloric acid (HCl, 36 - 38%) were purchased from Mallinckrodt Baker BV (Deventer, The Netherlands). *Di*-sodiumhydrogenphosphate 2-hydrate (99%), sodium acetate trihydrate (99.5 - 101.0%), *ortho*-phosphoric acid (85%) and trichloroacetic acid (TCA, > 99.5%) were from Merck. Sodium hydroxide (> 99%) was from Boom (Meppel, The Netherlands). CH_3OH-d_4 was from C.E. Saclay (Gif-Sur-Yvette, France).

5.2.2 Plant cell cultures

C. roseus line A12A2 was grown in M&S medium (Murashige and Skoog, 1962) without growth hormones, whereas *C. roseus* line A11 was grown in Gamborg B5 medium (Gamborg *et al*., 1968) containing NAA (1.86 mg/L). Both media were supplemented with 2% of D(+)-glucose monohydrate as the carbon source. The cells were grown in 250 mL-Erlenmeyer flasks containing 100 mL medium and cultivated at 24 (± 1) °C under continuous light (500 - 1500 lux), on a shaker at 100 rpm. The lines A12A2 and A11 were subcultured every week and every 3 weeks respectively by addition of the same volume of fresh medium to the cell cultures.

5.2.3 Elicitation

Pythium aphanidermatum (Edson) Fitzpatrick CBS 313.33 was used as an elicitor. This fungus was maintained on malt extract agar plate medium, at 25 °C, in the dark and subcultured every week. The solid culture was aseptically cut to pieces and two pieces (each about 1 cm^2) were transferred into a 250-mL Erlenmeyer flask containing 100 mL M&S liquid medium with 3% sucrose. This culture was then cultured at 27 °C on a shaker at 100 rpm for 7 days. It was then sterilized in an autoclave and subsequently filtered (to separate extract from broken cells/mycellia) under aseptic conditions. The filtrate was used as the elicitor. Ten or 20 ml of the *Pythium* extract was added to 100 mL of 5 days old *C. roseus* suspension cells. The cultivation conditions for the treated cultures were the same as that for culture maintenance. The elicited cells were harvested 24 h and/or 48 h after treatment by vacuum filtration using a P2 glass filter to separate the cells from medium. The cells were frozen in liquid nitrogen and used for the SA extraction.

5.2.4 Salicylic acid extraction

Non-elicited- and elicited cells of *C. roseus* A11 and A12A2 were weighed and homogenized using a mortar and pestle in the presence of liquid nitrogen. For 10 g of homogenized material (placed in a 50 mL-analytical tube), 2 x 25 mL of 100% MeOH were used for the extraction. The solid phase was separated from the liquid phase by a centrifugation (2500 rpm, 15 ºC, 10 min) using a Varifuge 3.0 R (Heraeus Sepatech, Germany). The MeOH extract was then evaporated using a rotary evaporator at maximum temperature 40 °C after the addition of 200 µl of 0.2 M NaOH.

Subsequently, the concentrated extract (± 5 mL) was acidified with 5 mL of 5% trichloroacetic acid (TCA) to pH 2.0, before partitioning with 10 mL of EtOAc-*n*-hexane (1:1), twice. In order to obtain SA from SA-glucoside (SAG), 3 mL of 8 M HCl was added to the TCA fraction resulting in pH 0 - 1 and placed in a water bath at 80 °C, 1 h, to hydrolyze the SAG. A partitioning with 15 mL of EtOAc-*n*-hexane (1:1) was performed twice to extract the liberated-SA. The non-polar extracts were pooled and evaporated using a rotary evaporator at room temperature. This extraction of SA was performed based on the method described by Verberne *et al*. (2002), therefore the free SA was extracted prior to acid hydrolysis for a separate quantitative analysis of free SA and SAG. However for extraction of total SA, acid hydrolysis can be performed on the concentrated MeOH extract prior to partitioning of the total SA. The concentrated extract (around 0.5 mL) was dissolved in 1-2 mL of 25 mM sodium phosphate (pH 7.0-7.5) : MeOH (90:10 v/v) before application to the IEC column.

5.2.5 Ion exchange chromatography

5.2.5.1 Application to SA standard

Ten grams, 2 g or 1 g of Dowex 1WX2 (100 mesh) were placed separately in glass columns (i.d. 1 cm) providing height of about 15, 3 and 1.5 cm respectively. The resin was rinsed and preconditioned with 25 mM sodium phosphate (pH 7.0 – 7.5) before loading with SA standard. This buffer complemented with 10% to 20% MeOH, was employed as the loading solvent. Elution of SA was performed using solvents containing different concentrations of HCl in aqueous AcCN (40 – 60%) or MeOH (15 - 60%). Fractions were collected, both from the washing steps (2 x 10 mL of the phosphate buffer without MeOH) and the elution steps (10 x 10 mL). Those obtained from the washing step were vortexed, centrifuged and analysed by HPLC. Also, the fractions of the elution step were vortexed and subsequently sampled (20 - 100 µl each). HPLC buffer was added to the samples, neutralized by the addition of 10 µl of 0.5 N NaOH (to adjust a pH 5 - 7 in the final volume of 500 µl of a sample) and centrifuged before injection into the HPLC system. A range of concentrations of SA standard solution (0.025 – 1.0 µg/mL) was used in every set of measurements.

5.2.5.2 Aplication to plant extracts

Samples of plant cell extracts containing different amounts of SA were loaded (using the same loading buffer mentioned as for experiments with pure SA) on separate columns to provide the systems with different ratios of the amounts of SA/resin. After washing each column with 30 x 10 mL of 25 mM sodium phosphate (pH 7.0 – 7.5), the SA was recovered with 10 x 10 mL of 0.3 M HCl in 60% AcCN. Preparation of the samples for HPLC analysis obtained from the washing step and the elution step was similar to that used for pure SA. The fraction containing most SA of the highest ratio of SA/resin system was evaporated using a rotary evaporator at room temperature. After evaporation, the remaining acidic aqueous extract (about 4 mL) was partitioned with 5 mL of EtOAc-*n*-hexane (1:1) twice. The a-polar phases were pooled and washed with the same volume of de-ionized water, twice. Sodium-sulphate was added to remove the remaining acid/water. Subsequently, the a-polar extract was separated from sodium-sulphate by a glass-pipette into a round-flask and evaporated at room temperature to dryness. The dried extract was re-dissolved in CH_3OH-d_4 for NMR analysis.

5.2.6 HPLC-analysis

HPLC analysis of SA was performed based on the system described by Verberne *et al*. (2002). The column, a Phenomenex column type LUNA 3µ C18 (2) 150 x 4.60 mm equipped with a SecurityGuard from Phenomenex (Torrance, CA, USA) was used. The mobile phase, 10 mM sodium acetate buffer in 10% MeOH (pH 5.5), was pumped using an LKB 2150 HPLC pump (Bromma, Sweden), at a flow rate of 0.80 ml/min. The injection (20 µl) of the samples was performed using a Gilson 234 auto-injector (Villiers Le Bel, France). The detection was carried out using a Shimadzu RF-10AxL spectrofluorometric detector (Tokyo, Japan), at an emission wavelength of 407 nm and an excitation wavelength of 305 nm, which was connected to a CR 501 Chromatopac printer (Shimadzu, Kyoto, Japan). The SA peak appears at around 13 min. Another HPLC equipment was also used to check the profile of impurities present in the washing fractions, consisting of a Waters 600 (Milford, MA, USA) pump, a Gilson sample injector model 231 with a Gilson dilutor model 401 (Villiers Le Bel, France), a Waters photodiode array (PDA) detector type 990 (Milford, MA,

USA) connected to a personal computer, and a Waters 5200 printer plotter (Milford, MA, USA).

5.2.7 ^{1}H-NMR analysis

NMR spectra were recorded on a Bruker AV 400 MHz spectrometer equipped with an Indy Silicon graphics computer. For each sample 128 scans were recorded with the following parameters: 0.17 Hz/point, pulse width (PW) = 4.0 μsec. (30 °C), and relaxation delay (RD) = 1.0 sec. FIDs were Fourier transformed with LB = 0.30 Hz. The NMR analyses were performed on the fraction containing most SA of the highest ratio of SA/resin experiment and of the SA standard dissolved in CH_3OH-d_4.

5.3 Results and discussion

5.3.1 Application to SA standard

A sample containing 100 μg of SA standard was applied to 10 g of the resin. There was no SA detected in the washing step but 10 mL of the elution solvent (0.1 M HCl in 15% MeOH, pH = 1) could elute only about 1% of the total SA applied. Changing the concentration of HCl to 0.25 M increased the SA eluted to about 2% of the total SA loaded per 10 mL elution. When the concentration of MeOH was increased to 40% (containing 0.25 M HCl), the SA amount increased to about 4.2% of the total SA loaded per 10 mL elution, and this amount was slightly improved to 4.6% when the HCl concentration was doubled (0.5 M). Increasing the concentration of MeOH improved the affinity of SA for the elution solvent, but not sufficiently for a good recovery. The SA elution profile obtained from the 10 g resin column pointed to a huge over-capacity of the column, which could be decreased to improve recovery. Therefore, we investigated the elution profile of 50 μg SA standard loaded on 1 g and 2 g of the resin employing 25 mM sodium phosphate (pH 7.0 – 7.5) as the washing solvent and 0.2 M HCl in 40% MeOH as the elution solvent. The result is presented in Figure 5.1 A.

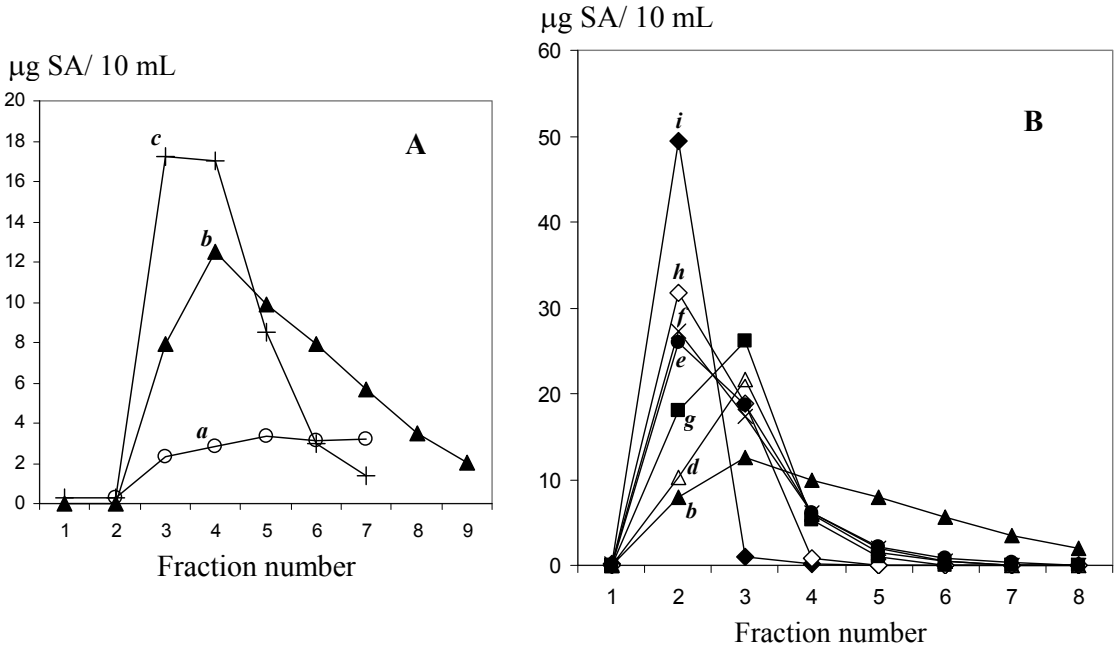

Figure 5.1. **A**: The elution profiles of SA standard applied on separate columns with different conditions. Ratio of amounts of SA/resin *a*: 10 µg/g (10 g resin used), *b*: 25 µg/g (2 g resin used), *c*: 50 µg/g (1 g resin used). The elution solvent employed was 0.25 M HCl in 40% MeOH for *a*, whereas 0.2 M HCl in 40% MeOH was used for *b* and *c*. Fraction no.1 – 2: the washing step. Fraction no. 3 – 9: the elution step. **B**: The elution profiles of SA standard applied on separate columns with ratio of amounts of SA/resin was 25 µg/g (2 g resin employed) using different concentrations of HCl and MeOH or AcCN, *b*: 0.2 M HCl in 40% MeOH, *d*: 0.2 M HCl in 60% MeOH, *e*: 0.3 M HCl in 60% MeOH, *f*: 0.4 M HCl in 60% MeOH, *g*: 0.2 M HCl in 40% AcCN, *h*: 0.2 M HCl in 60% AcCN, *i*: 0.3 M HCl in 60% AcCN. Fraction no.1: the washing step. Fraction no. 2 – 8: the elution step. The resin was Dowex 1WX2, 100 mesh and the washing solvent was 25 mM sodium phosphate (pH 7.0 – 7.5).

Since bigger ratio of amounts of SA/resin seemed to provide the most efficient elution, we investigated the influence of MeOH concentration on the SA (50 µg) elution profile of 1 g of the resin (data not shown). It was clear that higher HCl and MeOH concentration provided a more efficient SA elution. Also, some experiments using 50 µg of SA standard applied on 2 g of the resin were done to study the elution profiles affected by different concentrations of HCl and MeOH or AcCN (Figure 5.1 B). The use of AcCN was considered because AcCN has almost the same polarity as MeOH, 5.8 and 5.1 respectively (Snyder, 1978) but a bigger eluotropic strength than that of MeOH. This provides AcCN as a better solvent modifier for analytes

compared to MeOH not only in RP-HPLC but also in some ion-exchangers, affinity phases and chiral phases due to the hydrophobic interaction between analytes and the adsorbents (Rizzi, 1998). Increasing only the concentration of HCl from 0.3 M to 0.4 M but not the concentration of MeOH did not improve the SA elution. Figure 5.1 B shows that for 50 μg of SA applied to 2 g Dowex 1WX2 (100 mesh), the affinity of SA to the elution solvent increased by higher concentration of HCl and of the organic solvent. Changing the organic solvent from MeOH to AcCN resulted in the highest level of SA present in the first 10 mL elution. The cross-linked polystyrene matrix of the resin might have hydrophobic interactions with the undissociated SA, from which the SA could be eluted more efficiently by AcCN than by MeOH.

5.3.2 Application to plant cell extracts

For 2 g Dowex 1WX2 (100 mesh) loaded with 50 μg SA standard, after washing with a 25 mM sodium phosphate pH 7.0 – 7.5, the elution solvent 0.3 M HCl in 60% AcCN provided the most efficient SA elution profile since almost all of the SA applied (97%), eluted in the first 10 mL elution. However, the amount of SA in the concentrated plant cell extract loaded on the column of this IEC system could not be measured precisely. Part of SA might not be dissolved in the loading buffer and remained captured in the non-soluble matrix providing a non-reliable determination of the total amount of SA (in this case, the total amount of SA before loading was always less than the recovery). Therefore, we determined the total amount of SA in the MeOH extract or in the non-polar extract before evaporation as 100%. Thus, the SA recovery is affected by the loss of SA not only from the washing step but also from the whole process including evaporation and sample loading. An experiment using an acid-hydrolysis extract of 33 g fresh weight (FW) of *C. roseus* A11 elicited-cells containing only 7.2 μg SA resulted in 69% SA recovery in the elution solvent. Using the same washing and elution solvents, some experiments employing different amounts of endogenous SA (in different extracts of *C. roseus* A12A2 elicited cells) applied per gram of the resin were performed to study the SA recoveries and elution profiles. The results are shown in Figure 5.2 A.

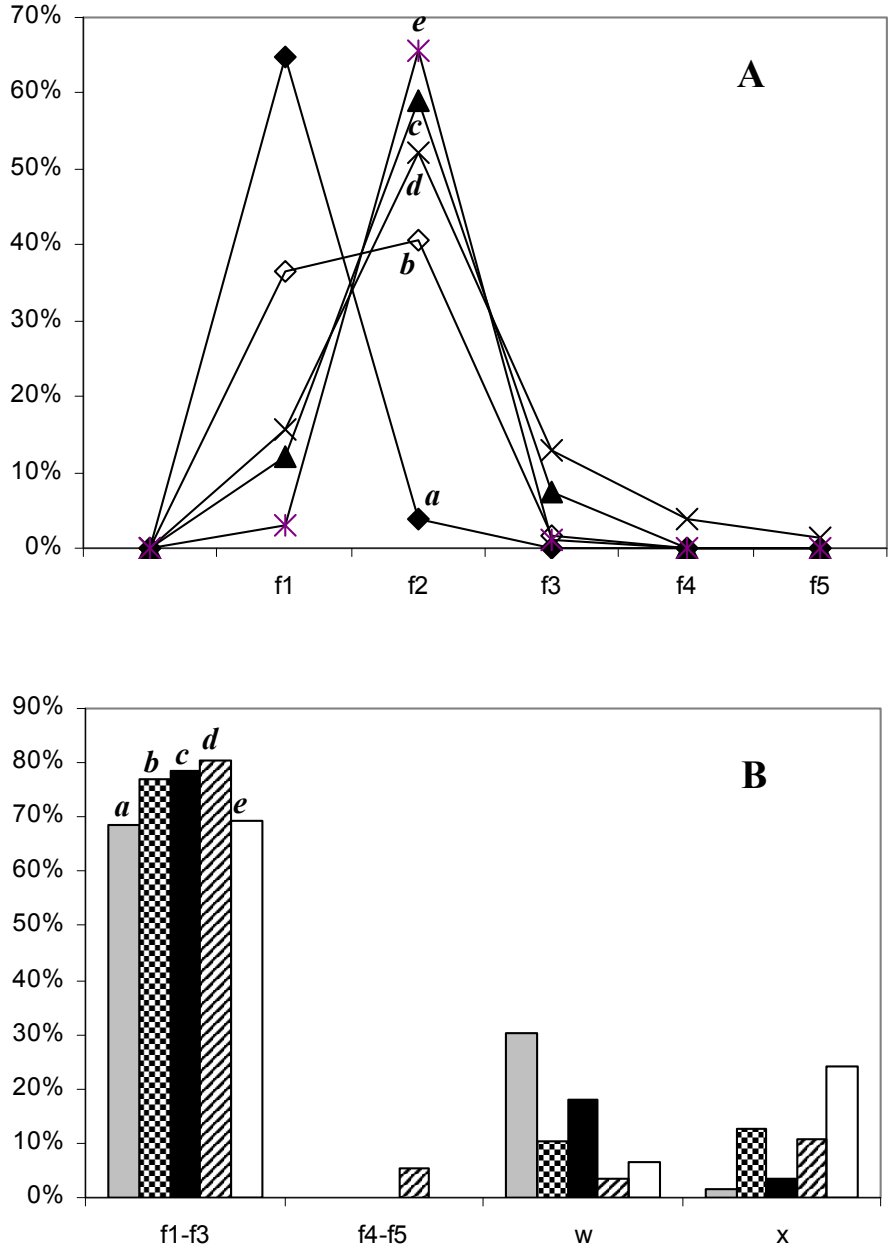

Figure 5.2. **A**: Effect of ratio of amounts of SA/resin on the elution profile of SA from plant extract. Expressed as % of SA recovered in the first 5 x 10 mL elution (fraction f1-f5) using Dowex 1WX2 100 mesh as the resin, 25 mM sodium phosphate pH 7.0 - 7.5 as the washing solvent and 0.3 M HCl in 60% AcCN as the elution solvent (*a*: 3.6 µg/g, *b*: 16.7 µg/g, *c*: 19 µg/g, *d*: 45 µg/g, *e*: 70 µg/g, the amounts of resin used in *a*: 2 g, *b*: 2.5 g, *c*: 3.5 g, *d*: 4 g, *e*: 4 g, the amount of SA before IEC application = 100 %). **B**: % of SA recovered in total first 3 x 10 mL elution (f1 – f3) and after the first 30 mL elution (f4 – f5), % of SA lost in the washing step (w) and % of SA lost in other steps (x).

The SA retention volume changes depending on the ratio of the amounts of SA/resin. Low ratio of SA/resin provided fast elution (in fraction 1), whereas late elution (in fraction 2) was obtained at high ratio of SA/resin. The application of an extract to the system provides a recovery of 69 - 80% (in total 3 x 10 mL first elution) from the whole purification process as shown in Figure 5.2 B. Not only a high SA recovery is necessary (because of the low level of SA produced by plants), but also a low degree of impurities after the separation is also important to allow a further NMR analysis of the purified SA.

Unfortunately, the impurities in the purified extracts were only checked by HPLC-fluorescence and not by HPLC-diode array detection (DAD). HPLC-DAD was only used to check the profile of impurities in the washing step, which showed many huge-peaks after 30 min in the chromatograms (data not shown). Despite of the selectivity of fluorescence detector, the fluorescence chromatograms of the crude extracts showed many peaks at 2 – 5 minutes, which were highly-reduced or often disappeared in the fluorescence chromatograms of the most SA-containing fractions after purification. One of those small peaks of impurities detected at around 2 - 5 minutes in the HPLC-fluorescence chromatogram of the most SA-containing fraction after purification was the peak of 2,3-dihydroxybenzoic acid (data not shown). This compound is present in a relatively large amount as compared to SA in a MeOH extract of *C. roseus* elicited cells (Budi Muljono, 2001). By increasing the volume of the washing buffer from 50 mL to 300 mL the impurities were almost completely removed as could be detected by the HPLC-DAD analysis of the last 10 mL of the washing step, or in the HPLC-fluorescence chromatogram of the major SA fractions of the purification method (data not shown). The ^{1}H-NMR spectrum shows that the IEC system used was relatively selective for SA. In a typical experiment, 65% of the SA was recovered in the second 10 mL elution of a plant extract containing 280 μg of total SA extracted from 200 g fresh weight *C. roseus* elicited cells (4 g of resin was used, providing SA/resin ratio of 70 μg/g). This amount of SA provided well detectable SA signals with a low level of impurities in the 400 MHz ^{1}H-NMR spectra (Figure 5.3).

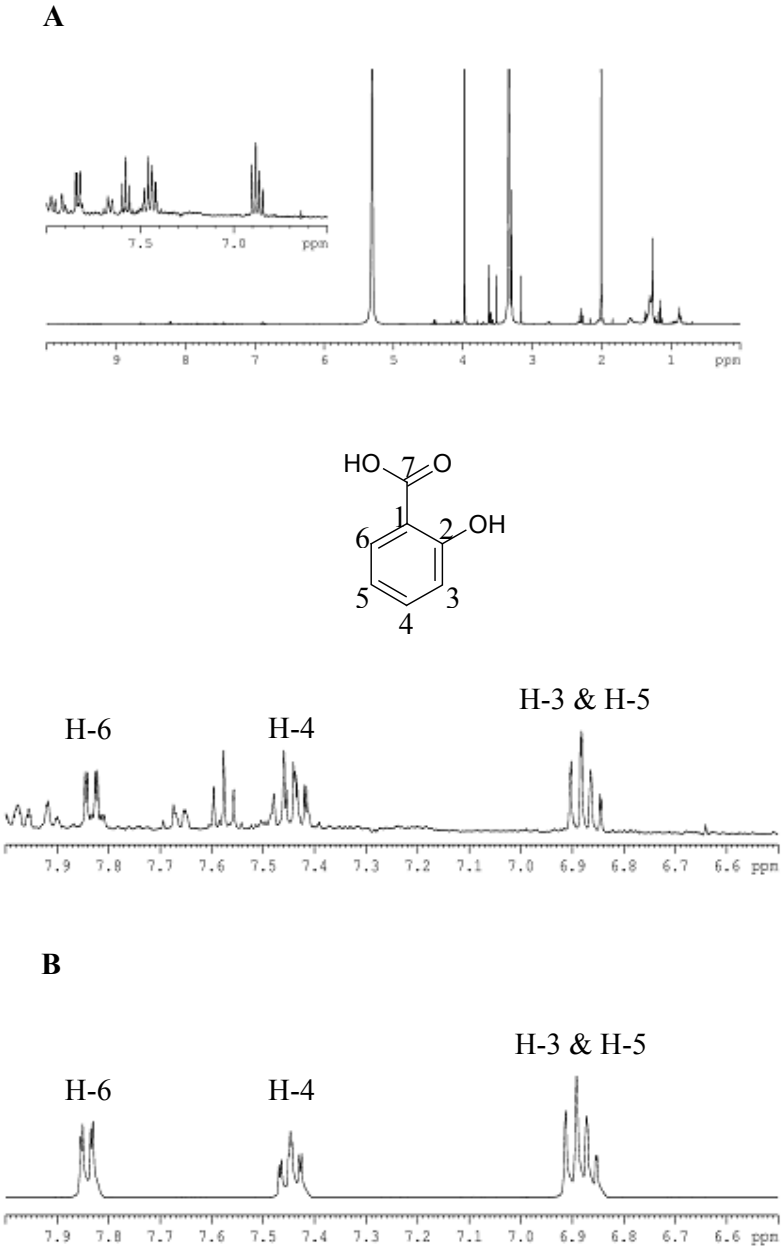

Figure 5.3. **A**: The ^{1}H-NMR spectra (CH_3OH-d_4) of the most SA-containing fraction after an ion exchange chromatographic separation (184 µg of endogenous SA determined by HPLC). **B**: The ^{1}H-NMR spectra of 180 µg of SA standard dissolved in CH_3OH-d_4.

5.4 Conclusion

An ion exchange chromatographic system consisting of 2 g of Dowex 1WX2 (100 mesh) as the resin, 25 mM sodium-phosphate pH 7.0 – 7.5 as the washing solvent and 0.3 M HCl in 60% AcCN as the counter ion solution that applied to 50 µg SA standard, provided an efficient SA elution profile since almost all SA (97%) eluted in the first 10 mL eluent. Depending on the ratio of the amounts of SA/resin, the SA retention volume changes. Low ratio of SA/resin provided fast elution (in fraction 1), whereas late elution (in fraction 2) was obtained at high ratio of SA/resin. The application of an extract to the system provides a recovery of 69 - 80% (in total 3 x 10 mL first elution) calculated from the whole purification process. Depending on the amount of SA in an extract, the amount of the resin can be determined to obtain the best elution profile with most of SA concentrated in one fraction. The amount of SA (184 µg) in the most SA-containing fraction after purification of a plant extract containing 280 µg of total SA extracted from 200 g FW *C. roseus* elicited cells (4 g resin was used, providing the ratio of SA/resin of 70 µg/g), provided well detectable SA signals with a low level of impurities in the 400 MHz -^{1}H-NMR spectra. This method is thus suited as a single step concentration and purification method of SA, as trace compound in *Catharanthus roseus* cell cultures.

Chapter 6

A retrobiosynthetic study of salicylic acid production in *Catharanthus roseus* cell suspension cultures

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Abstract

A feeding experiment using [1-^{13}C]-D-glucose to *Catharanthus roseus* (L.) G.Don cell suspension cultures followed by elicitation with *Pythium aphanidermatum* extract was performed in order to study the salicylic acid (SA) biosynthetic pathway and that of 2,3-dihydroxybenzoic acid (2,3-DHBA) as a comparison. A strongly labeled C-7 and a symmetrical partitioning of the label between C-2 and C-6 would occur if SA was synthesized from phenylalanine. In case of the isochorismate pathway, a relatively lower incorporation at C-7 and a non-symmetrical incorporation at C-2 and C-6 would be obtained. Relatively high- and non-symmetrical enrichment ratios at C-2 and C-6, and a lower enrichment ratio at C-7 were observed in both SA and 2,3-DHBA detected by ^{13}C-NMR inverse gated spectrometry leading to the conclusion that the isochorismate pathway is responsible for the biosynthesis of both compounds. The resultant of different enrichment ratios of the carbons in SA and 2,3-DHBA indicates the use of different isochorismate pools, which means that their biosynthesis is separated in time and/or space.

Keywords: salicylic acid biosynthesis, 2,3-dihydroxybenzoic acid, isochorismate pathway, phenylalanine pathway, *Catharanthus roseus* cell suspension cultures, fungal elicitation

6.1 Introduction

Salicylic acid (SA) is not only a precursor of siderophores (e.g enterobactin and pyocheline) produced by microorganisms for uptake of Fe^{3+} in an environment with highly insoluble $Fe(OH)_3$, but it is also an important signal compound for inducing systemic acquired resistance (SAR) in plants. Up to now there are two possible pathways known to lead to SA in living organisms. The first one is the microbial SA pathway, which consists of two steps. The first step in the microbial synthesis is the conversion of chorismate into isochorismate by isochorismate synthase (ICS, EC 5.4.99.6), and the second step is the conversion of isochorismate into SA by isochorismate pyruvate-lyase (IPL). The second pathway for SA is the phenylalanine pathway, which has been long thought to be the responsible pathway for SA synthesis in plants. However, the complete pathway has not been resolved yet. In this pathway, SA is the product of hydroxylation of benzoic acid (BA) at the *ortho* position by benzoic acid 2-hydroxylase. Benzoic acid is synthesized by the chain shortening of cinnamic acid either through a *β*-oxidative pathway or a non-oxidative pathway (reviewed by Verberne *et al*., 1999; Mustafa and Verpoorte, 2005).

Verberne *et al*. (2000) postulated the presence of the isochorismate pathway for SA biosynthesis in plants. They successfully overexpressed the microbial SA pathway in tobacco. Wildermuth *et al*. (2001) found indirect evidence for the existence of the SA isochorismate pathway in *Arabidopsis thaliana*. The *Arabidopsis sid2* mutant that was unable to produce chloroplast-localized ICS1 exhibited a remarkable decreased-level of SA after an infection and a reduced-resistance against pathogenic fungi or bacteria. The phenylalanine pathway or the presence of another *ICS* gene was further proposed to be responsible for the basal level of SA (Wildermuth *et al*., 2001). However, Chong *et al*. (2001) showed that the SA accumulation in elicited tobacco cells required *de novo* BA synthesis from *trans*-cinnamic acid, though, instead of free BA, the benzoyl-glucose was likely to be the main intermediate in the SA biosynthesis. The metabolic pathway from *trans*-cinnamic acid to SA via benzoic acid is also involved in the stress-induced flowering of *Pharbitis nil* (Hatayama and Takeno, 2003).

In *Catharanthus roseus* cell suspension cultures, the levels of SA and 2,3-DHBA increase after elicitation with fungal cell-wall preparations and are in parallel with an

increased-activity of the enzyme isochorismate synthase (Moreno *et al*. 1994a; Budi Muljono *et al*., 2002). A retrobiosynthetic study with *C. roseus* suspension cells fed with [1-^{13}C]glucose has shown the intermediacy of isochorismate in 2,3-DHBA biosynthesis rather than phenylalanine (Budi Muljono *et al*., 2002). Hence, the presence of the isochorismate pathway leading to SA in *C. roseus* seems also an alternative. In the present study, we performed a [1-^{13}C]glucose feeding experiment with a *C. roseus* cell suspension culture, in order to identify the SA pathway employed by the cells after elicitation with *Pythium aphanidermatum* extract as this fungal cell wall preparation increased the SA level in *C. roseus* cells (Budi Muljono, 2001).

The [1-^{13}C]glucose will be broken down through glycolysis or Embden-Meyerhof-Parnas (EMP) pathway into two 3-carbon units (glyceraldehyde-3-phosphate/PGAL) in the cytosol leading to the precursors of the shikimate pathway, phosphoenol-pyruvate (PEP) and erythrose-4-phosphate (E4P) as shown in Figure 6.1. This pathway is common to both prokaryotes and eukaryotes. However, there are also alternative pathways for converting hexoses (C_6) into trioses (C_3) named the pentose-phosphate pathway and the Entner-Doudoroff pathway, which are found in many organisms but can not be considered universal (Berg *et al*., 2002). The (C_3) pyruvate is broken down into a (C_2) acetyl-CoA (with CO_2 as by-product). The acetyl-CoA is transported into mitochondria and added onto oxaloacetate to enter the Krebs cycle for producing energy (GTP/ATP) and the enzyme co-factors NADH and $FADH_2$, which are needed for many other metabolic pathways including glycolysis. The starting and ending molecule in the Krebs cycle is oxaloacetate, which can be converted again into pyruvate. The Krebs cycle is of central importance in all living cells that utilize oxygen (aerobic organisms) as part of cellular respiration. Thus, the ^{13}C isotope from [1-^{13}C]glucose will be incorporated in the shikimate pathway into chorismate through several routes that produce differently labeled PEP and E4P.

If the precursors [3-^{13}C]PEP and [4-^{13}C]E4P from direct glycolysis of the labeled glucose are predominant in the flux to the shikimate pathway, chorismate will be mainly labeled at C-2 (from PEP) and C-6 (from E4P) (Rajagopalan and Jaffe, 1993) as shown in Figure 6.2. As a consequence, if SA is formed via the isochorismate pathway, there will be a low labeling at the carboxyl group (C-7) and the labeling at C-2 and C-6 positions will not be equal as isochorismate is not symmetric.

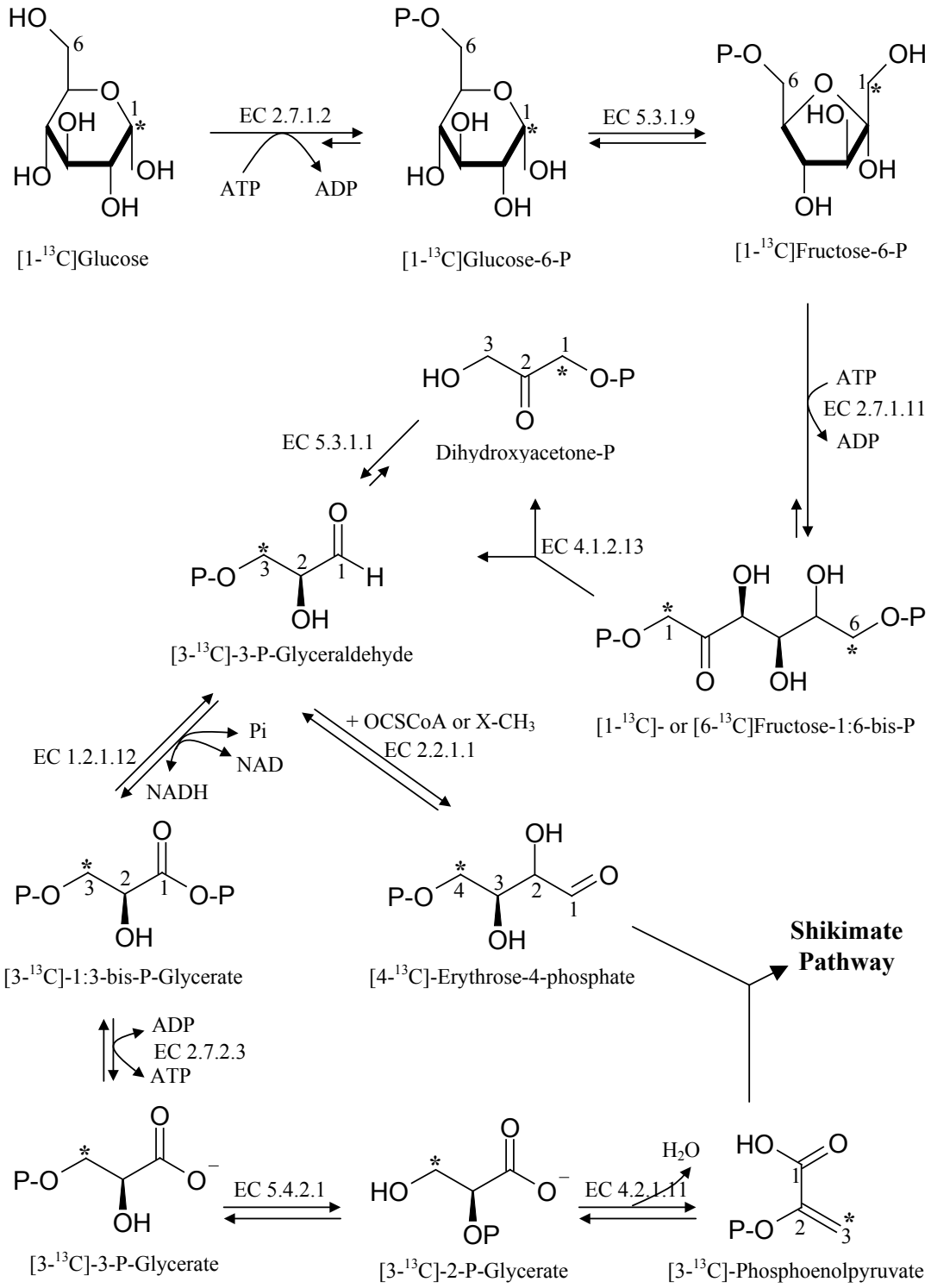

Figure 6.1. Glycolysis/ Embden-Meyerhof-Parnas

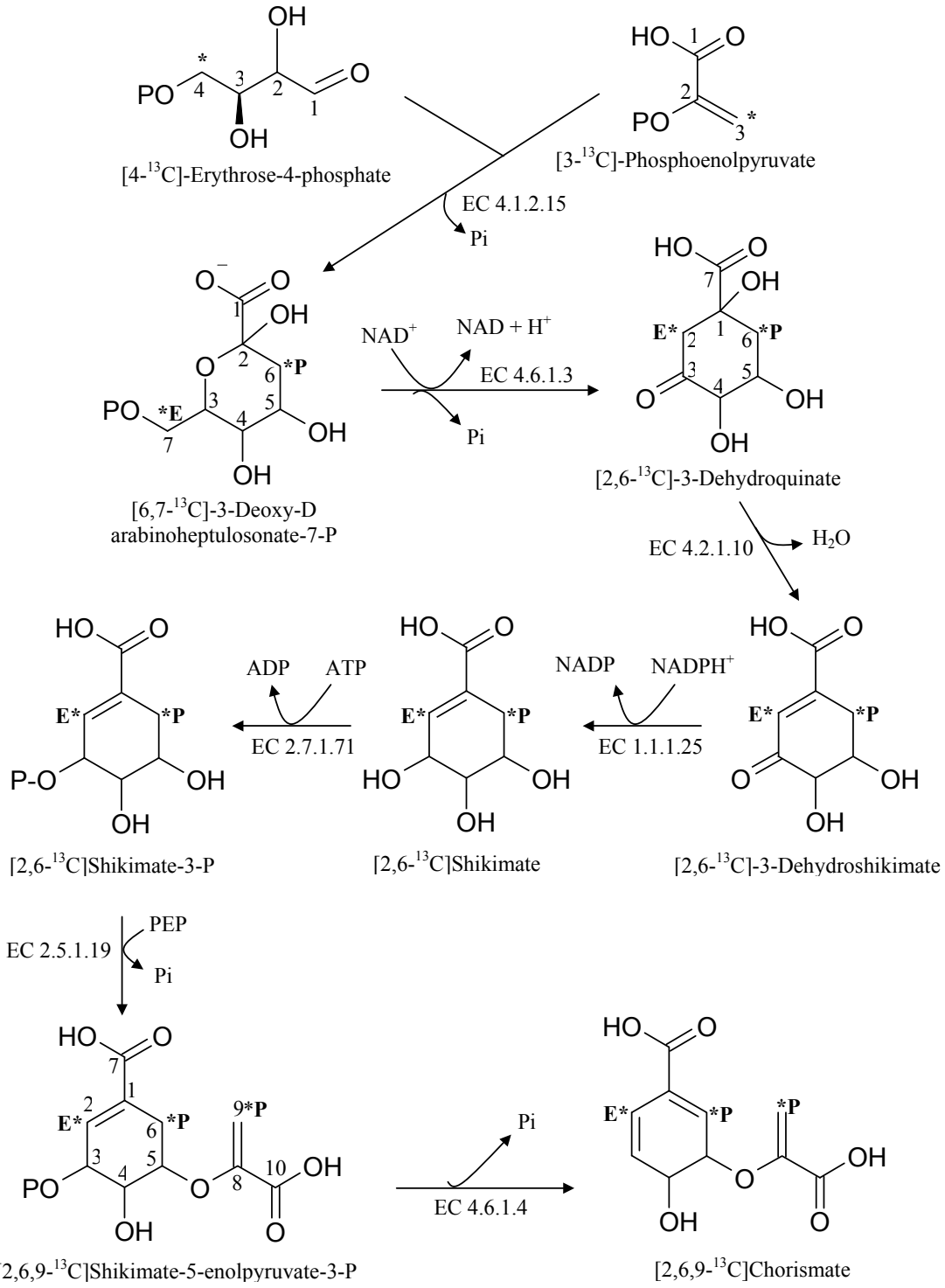

Figure 6.2. The shikimate pathway. E*: label from erythrose-4-phosphate, P*: label from phosphoenolpyruvate.

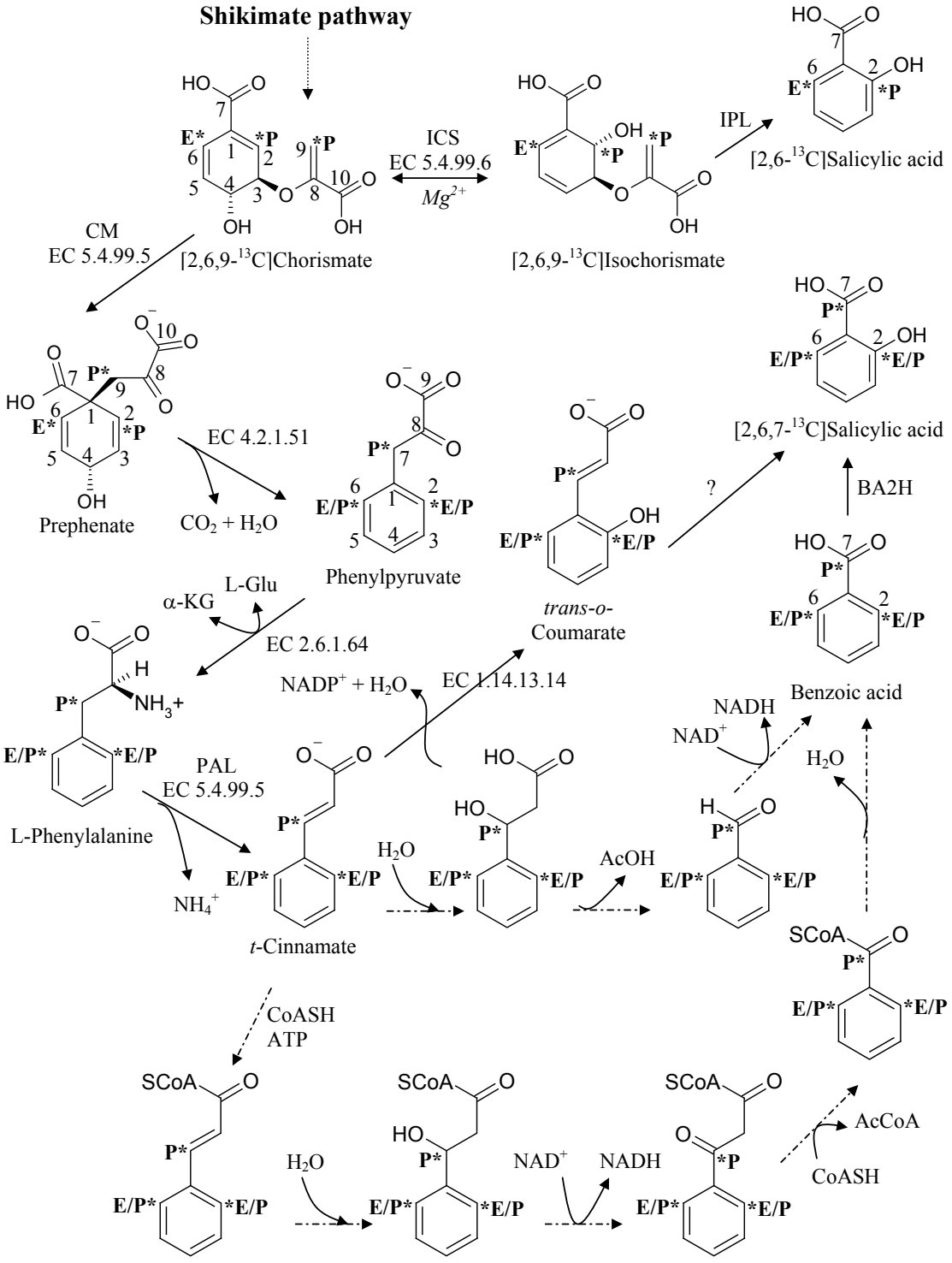

Figure 6.3. Salicylic acid biosynthetic pathway. CM= Chorismate mutase; ICS= Isochorismate synthase; IPL= Isochorismate pyruvate-lyase; PAL= Phenylalanine ammonia-lyase; BA2H= Benzoic acid 2-hydroxylase; *E: label from erythrose-4-phosphate; *P: label from phosphoenolpyruvate.

However, if SA is synthesized from the phenylalanine pathway, a high incorporation will occur in the carboxyl group (C-7) via the C-9 of chorismate, originating from C-3 of PEP (Figure 6.3). Though all carbons in the ring of chorismate will be labeled differently, the labeling of phenylalanine at the positions C-2 and C-6 as well as at the positions C-3 and C-5 will be the same since the aromatic ring of phenylalanine is symmetric along the C-1/C-4 axis (Werner *et al*., 1997). These pairs of carbons are chemically equivalent and they are not distinguishable in the NMR spectra. Also, the chance of oxidation of the *ortho*-positions on either side of the propane side chain leading to SA is equal. Consequently, similar labeling at the positions C-2 and C-6 will be found in the phenylalanine-derived SA.

These clearly different labeling patterns of SA provided by different pathways were used in this study to identify the SA pathway employed by *C. roseus* cells.

6.2 Materials and methods

6.2.1 Plant cell cultures

Catharanthus roseus line A12A2 was grown in Murashige and Skoog (M&S) medium (Murashige and Skoog, 1962) without growth hormones supplemented with 2% of D(+)-monohydrate glucose as the carbon source. The cells were grown in 250 mL-Erlenmeyer flasks each containing 100 mL medium and cultivated at 24 (± 1) °C under continuous light (500 - 1500 lux), on a shaker at 100 rpm. The suspension cultures with labeled-glucose was obtained by subculturing on the M&S medium containing no growth hormone supplemented with 2% of [1-^{13}C]-D(+)glucose.

6.2.2 Elicitor

Pythium aphanidermatum (Edson) Fitzpatrick CBS 313.33 was used as an elicitor. This fungus was maintained on malt extract agar medium, at 25 °C, in the dark and subcultured every week. Aseptically, the solid culture was cut to pieces. Two pieces (each about 1 cm^2) were transferred into a 250-mL Erlenmeyer flask containing 100 mL M&S liquid medium without growth hormone and supplemented with 3% sucrose. This culture was cultivated at 27 °C on a shaker at 100 rpm for 7 days, sterilized in an autoclave (120 °C, 20 min) and subsequently filtered to separate

extract from broken mycelia under aseptic conditions. The filtrate was used as the elicitor.

6.2.3 Elicitation and harvesting cells

Twenty ml of the *Pythium* extract was added to 100 mL of the 5-days old *C. roseus* suspension cells grown in labeled-glucose medium. The cultivation conditions for the treated cultures were the same as that for plant cell culture maintenance. The elicited cells were harvested 24 h after treatment by vacuum filtration using a P2 glass filter to separate the cells from the medium. The cells were rinsed on the filter with de-ionized water, collected, frozen in liquid nitrogen and freeze-dried for 72 h before extraction with MeOH.

6.2.4 Chemicals

6.2.4.1 Chemicals used for the medium of cell suspension cultures

The chemicals used in Macro Murashige & Skoog (M&S) salts were: $CaCl_2$ (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 $MgSO_4$ exsiccatus BP was from OPG Farma BUVA B.V.(Uitgeest, The Netherlands). The chemicals used in Micro M&S salts were: H_3BO_3, $MnSO_4.H_2O$, Na_2EDTA, $ZnSO_4.7H_2O$ (Merck) and $FeSO_4.7H_2O$ (Brocades-ACF groothandel NV, Maarssen, The Netherlands) were dissolved in one solution, whereas $CoCl_2.6H_2O$, $CuSO_4.5H_2O$, KI and $NaMoO_4.2H_2O$ (Merck) were dissolved in another solution due to the problem of solubility. Glycine (99.7%) and nicotinic acid (99.5%) were purchased from Merck. D(+)-Glucose (> 99.0%) was obtained from Fluka Chemie (Buchs, Germany) whereas [1-^{13}C]-D-glucose (> 99%, with > 99% atom 1-^{13}C) was from Campro Scientific (Veenendaal, The Netherlands). myo-Inositol (> 99.0%) was from Duchefa Biochemie (Haarlem, The Netherlands). Pyridoxine-HCl was from Sigma-Aldrich Chemie (Steinheim, Germany) and thiamine-*di*-HCl was from Janssen Chimica (Geel, Belgium).

6.2.4.2 Chemicals used for extraction, purification and analysis of salicylic acid

Dowex 1WX2 (100 mesh) and salicylic acid were purchased from Sigma-Aldrich, (St. Louis, MO, USA). Acetonitrile (> 99.8%), ethyl acetate (> 99.8%) and methanol (> 99.8%) were from Biosolve B.V. (Valkenswaard, The Netherlands). Acetic acid

(100%), ammonia, *n*-hexane (> 99%) and hydrochloric acid (36 - 38%) were purchased from Mallinckrodt Baker B.V. (Deventer, The Netherlands). *Di*-sodiumhydrogenphosphate 2-hydrate (99%), *ortho*-phosphoric acid (85%) and trichloroacetic acid (> 99.5%) were obtained from Merck. Sodium hydroxide (> 99%) was from Boom (Meppel, The Netherlands). CH_3OH-d_4 was from C.E. Saclay (Gif-Sur-Yvette, France).

6.2.5 2,3-DHBA extraction

Eight hundred mL of medium harvested from the 1st batch of labeling experiments was acidified with phosphoric acid (85%) to obtain a pH 4.0 and subsequently extracted with 800 mL of ethyl acetate, twice. The ethyl acetate phases were pooled, rinsed with the same volume of de-ionized water, sodium-sulphate was added to remove the residual water and the ethyl acetate was evaporated using a vacuum rotary evaporator at room temperature till dryness. The dried extract was re-dissolved in 1 mL of CH_3OH-d_4 for NMR analysis.

6.2.6 Salicylic acid extraction

Twenty-four hours-elicited cells of A12A2, harvested from 9 or 10 Erlenmeyer-flasks (each containing 100 mL) of cell suspension culture, were transferred to the same number of 50mL-tubes and freeze-dried for 72 h. Twenty-five mL of 90% MeOH was added to the dried material in each tube followed by vortexing, sonication (20 min) and maceration (24 h). The mixture was then centrifuged (at 2500 rpm, 15 ºC, 10 min) using a Varifuge 3.0 R (Heraeus Sepatech, Germany) and the supernatant was collected. The pellet was extracted again using 20 mL of 100% MeOH followed by vortexing, sonication (20 min) and maceration (24 h). The mixture was centrifuged: the supernatant from this second maceration was combined with the first one, adjusted to a volume of 35 – 45 mL/tube from which a sample was taken to determine the SA level by HPLC. All of the MeOH extracts were pooled, subsequently 1 mL of 0.2 N NaOH was added and the solvent was evaporated to almost dry, using a vacuum rotary evaporator at a temperature maximum of 40 ºC. To the concentrated extract (about 1 mL) 10 - 15 mL of 10% trichloroacetic acid (TCA) was added resulting in pH 1.0 - 1.5 and subsequently partitioned with 3 x 10 mL of EtOAc - *n*-hexane (1:1). The non-polar phase (containing free SA) was collected. To the TCA fraction subsequently 5 mL of 8 N HCl was added (resulting in pH 0) and

placed in a water-bath (at 80 ºC, 1 h) to hydrolyze the SA-glucoside. The liberated SA was extracted with 3 x 15 mL of EtOAc - *n*-hexane (1:1). Both non-polar extracts containing SA were evaporated separately using a vacuum rotary evaporator at room temperature till dryness. Each extract was re-dissolved in 2 mL of 25 mM sodium-phosphate (pH 7.0 – 7.5) containing 10 – 20% MeOH before application to an ion exchange chromatography (IEC) column.

6.2.7 HPLC-fluorescence analysis

HPLC analysis of SA was performed based on the system described by Verberne *et al*. (2002) with a modification in the HPLC buffer. The column, a Phenomenex column type LUNA 3µ C18 (2) 150 x 4.60 mm equipped with a SecurityGuard from Phenomenex (Torrance, CA, USA) was used. The mobile phase, 0.2 M ammonium acetate buffer in 10% MeOH (pH 5.5), was pumped using an LKB 2150 HPLC pump (Bromma, Sweden), at a flow rate of 0.80 ml/min. The injection (20 µl) of the samples was performed using a Gilson 234 auto-injector (Villiers Le Bel, France). The detection was carried out using a Shimadzu RF-10AxL spectrofluorometric detector (Tokyo, Japan), at an emission wavelength of 407 nm and an excitation wavelength of 305 nm, which was connected to a CR 501 Chromatopac printer (Shimadzu, Kyoto, Japan). A range of concentrations of SA standard was used for every set of quantitative measurements. The SA peak appears at around 11 min.

6.2.8 Purification using ion exchange chromatography

Purification of SA by ion exchange chromatography was performed based on the system described in Chapter 5 of this thesis. The free SA extract or extract obtained from the acid hydrolysis was dissolved in 25 mM sodium phosphate buffer (pH 7.0 – 7.5) containing 10 – 20% MeOH and applied onto the column containing (2.5 – 4.0 g) Dowex 1WX2, 100 mesh (Sigma-Aldrich). Three hundred mL of 25 mM sodium phosphate buffer (pH 7.0 - 7.5) were used as the washing buffer to separate SA from most of the impurities, resulting in 30 fractions. To recover SA from the resin, 100 mL of 0.3 M HCl in 60% acetonitrile (resulting in 10 fractions) were used as the counter ion solution. Samples were taken out from the washing fractions and from the acidic fractions for HPLC analysis. The acidic fractions containing a relatively high amount of SA were pooled and evaporated. Subsequently, partitioning with 4 x 10 mL

of EtOAc - *n*-hexane (1:1) was performed with the remaining acidic-aqueous part. The non-polar fractions were pooled, evaporated in a vacuum-rotary evaporator at room temperature till dryness, and re-dissolved in 1 mL of MeOH.

6.2.9 Purification using gel exclusion chromatography

Thirty grams of Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) were suspended in 100% MeOH and subsequently put into a glass column with a diameter of 2 cm. The length of the column packed with the stationary phase was 33.5 cm. The stationary phase was rinsed with 200 mL of (degassed) 100% MeOH before application of the extract. An IEC-purified extract (in 1 mL of MeOH) was applied on the column and fractionated (each 10 mL) using 300 mL of (degassed) 100% MeOH. Samples were taken from the fractions and analyzed by HPLC. The fraction containing SA was evaporated and re-dissolved in 1 mL of CH_3OH-d_4 for NMR analysis.

6.2.10 NMR analysis

^{1}H-NMR and ^{13}C-NMR spectra were recorded in CH_3OH-d_4 using a Bruker DMX 600 MHz spectrometer, whilst the HMBC and J-resolved ^{1}H-NMR spectra were recorded in CH_3OH-d_4 by a Bruker AV 500 MHz spectrometer. For inverse gated ^{13}C-NMR analysis of enriched SA and -2,3-DHBA, 36864 scans were recorded with the following parameters: pulse width (30º, 13.0 μsec), DS = 4, DW = 13.9 μsec, AQ = 1 sec and relaxation delay (Dl) = 2.0 sec. FIDs were Fourier transformed with LB = 2.20 Hz and SI = 128 kHz.

6.3 Results and discussion

Amongs four different *C. roseus* cell lines, the A12A2 cell suspension culture (grown in M&S medium without growth hormone) was chosen for the labeling experiment since it provided the highest level of free SA (1.0 – 2.5 μg/ g fresh weight cells) after elicitation with *Pythium aphanidermatum* extract (Chapter 3). The cells were grown in M&S medium with a final concentration of 1% [1-^{13}C]-D-glucose in the medium. This level of labeled glucose is relatively high in order to minimize the possible dilution of the labeled precursors due to the remaining non-labeled precursors present in the cells from the previous subculturing with non-labeled

glucose. The attempt to reduce dilution is necessary since the experiment deals with a plant hormone (signaling compound), which is produced by plant cells in low amounts only (ppm level). Moreover, final yields are affected by loss of the compound during the purification. The duration of feeding (5 days) before the elicitation, was aimed at increasing the biomass to produce a higher level of labeled-SA. Budi Muljono (2001) investigated the growth of the *C. roseus* (suspension) cells and found that the maximum of the biomass was reached on the fifth day after subculturing. Concerning SA, we found the highest level of total SA in the cells 24 h after elicitation with 20 mL *Pythium* extract compared with cells 48 h after elicitation, suggesting that the catabolism of SA 48 h after elicitation is higher than *de novo* biosynthesis.

Three independent labeling experiments were performed on batches of the A12A2 cell suspension cultures. Each batch contained 9 – 10 Erlenmeyer-flasks of 100 mL cell culture, which provided around 6 – 9 g dry weight cell material (about 4 – 5% of the fresh weight cells). The levels of free SA were determined by HPLC in the MeOH extract (obtained from the first step of the extraction protocol), in the fractions obtained from the IEC and from the second purification using a Sephadex LH-20 column (Table 6.1). The determination of the free SA level of batch 3 before IEC was performed in the MeOH extract collected from 48 h maceration (total 165 μg). By continuing maceration with a fresh volume of MeOH till 96 h, the amount of total free SA extracted increased. Some SA (56 μg) was lost most probably in the process before the purification with gel exclusion chromatography.

In the ^{1}H-NMR spectra of the extract obtained from the first purification using IEC we could detect the SA peaks quite well among the peaks of some remaining impurities (e.g. 2,3-DHBA). However, in the 2-D NMR spectra (HMQC, HMBC and J-Resolved) of this extract the SA signals were not as pronounced, suggesting the need of a second purification step before ^{13}C-NMR analysis. For this, Sephadex LH-20 resin was used since its separation mechanism is not only based on the size/ molecular weight (MW) of the compounds in the extract but also on adsorption, in which different polarities of some compounds with almost similar molecular weight (e.g. different in one hydroxyl group) can result in different retention.

Table 6.1. Amounts of SA harvested. * For batch 1: free SA and SA after acid-hydrolysis were pooled and loaded on the column. DW: dry weight, FW: fresh weight, IEC: ion exchange chromatography.

Batch number	g DW cells	% to FW	Amounts of Free SA (μg)				
			Before IEC	After IEC		After fractionation by Sephadex LH-20 *	
				In the washing step	In the elution step	In fraction no. 11	In fraction no. 12
1	6.47	5.1	181	6	155	109	48
2	7.85	4.7	66	12	52	Not performed	Not performed
3	9.53	4.2	> 165	17	190	85	49

Besides, this method provides a good recovery (almost 100%, data not shown). Elution with 300 mL of 100% MeOH to the (IEC-purified) extract of the first batch provided 30 fractions (each 10 mL), in which the fraction number 11 and 12 contained 109 μg and 48 μg SA respectively. The inverse gated ^{13}C-NMR method was chosen to analyze fraction-11. Figure 6.4 A shows the ^{13}C-NMR spectra of fraction-11 of batch 1. Due to the lower level of SA and the level of impurities (detected by ^{1}H-NMR), the fraction no.12 (of batch 1 and batch 3) were not analyzed further. Also, batch 2 that provided only around 66 μg of free SA (as detected before purification) was not analyzed. An inverse gated ^{13}C-NMR analysis was also performed on the fraction-11 of batch 3 containing around 85 μg SA (Figure 6.4 B), however, the noise-level was higher than that of the batch 1 and therefore would not be used for a comparison or quantitative analysis.

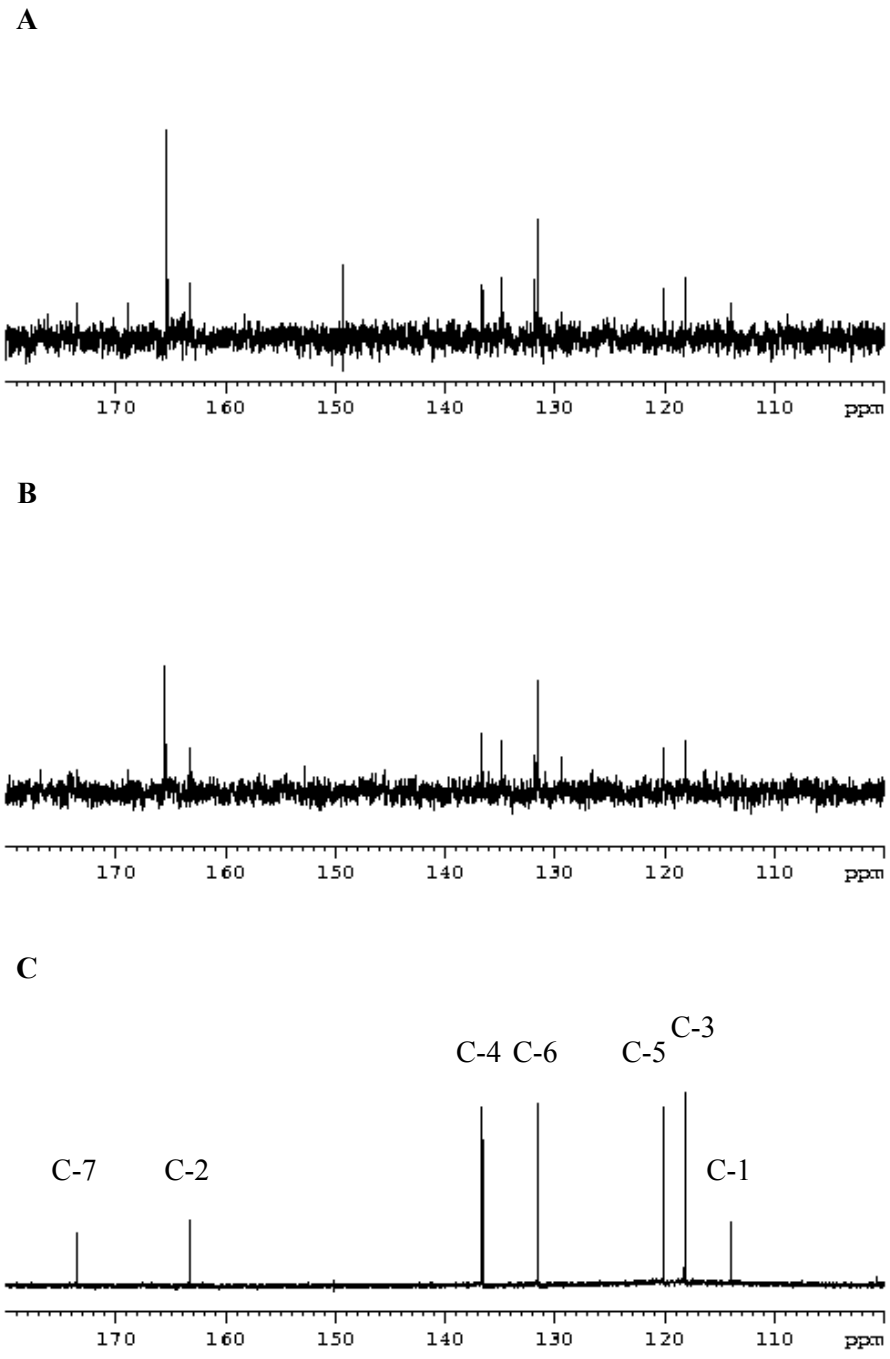

Figure 6.4. ^{13}C-NMR inverse gated spectra (CH_3OH-d_4) of purified extracts of *Catharanthus roseus* elicited cells containing labeled/enriched SA (A: batch 1 and B: batch 3) and of SA standard (C, 10 mg/mL).

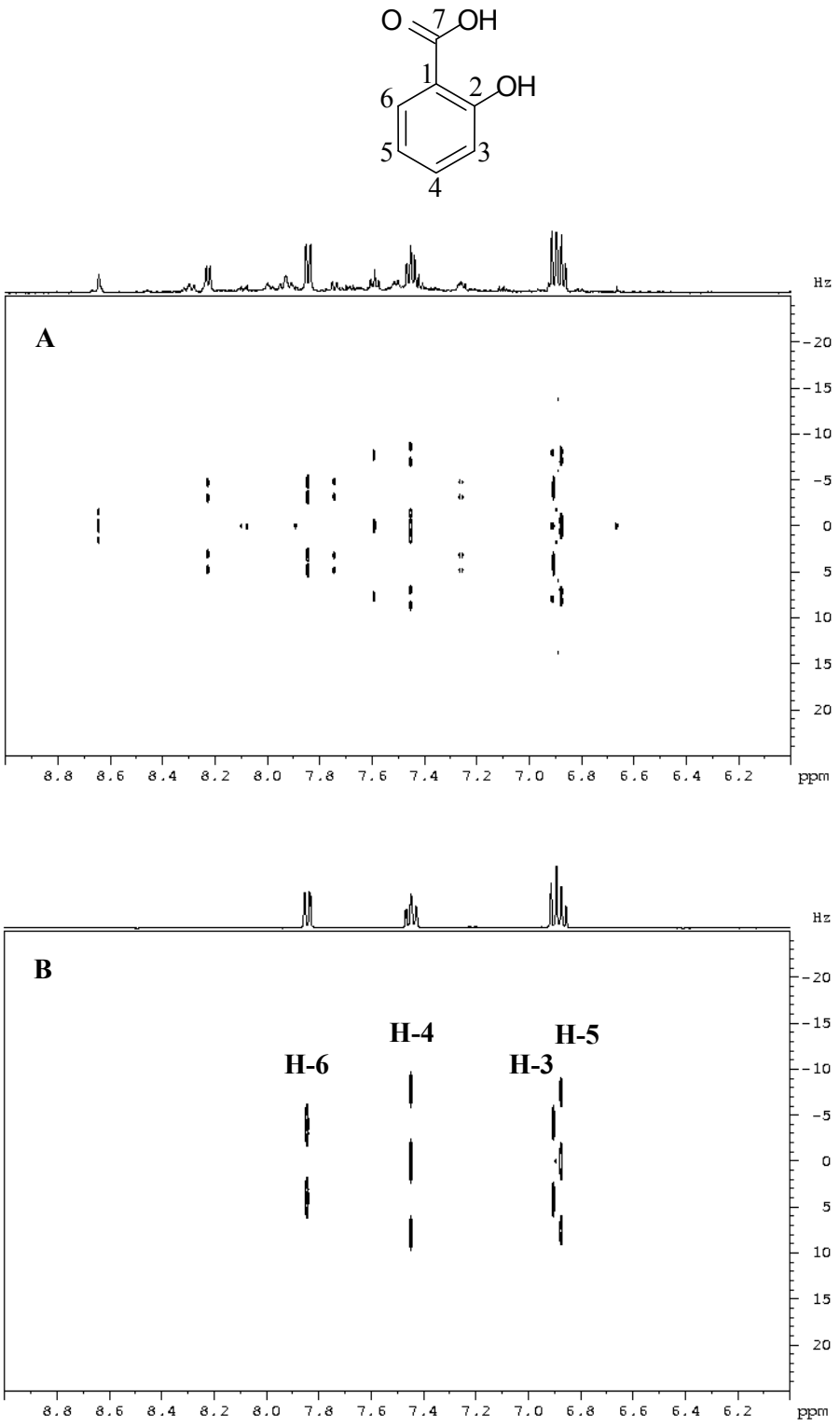

Figure 6.5. J-resolved ^{1}H-NMR spectra of purified extract of *Catharanthus roseus* elicited cells batch 1 containing labeled/enriched SA (A) and of SA standard (B) dissolved in CH_3OH-d_4.

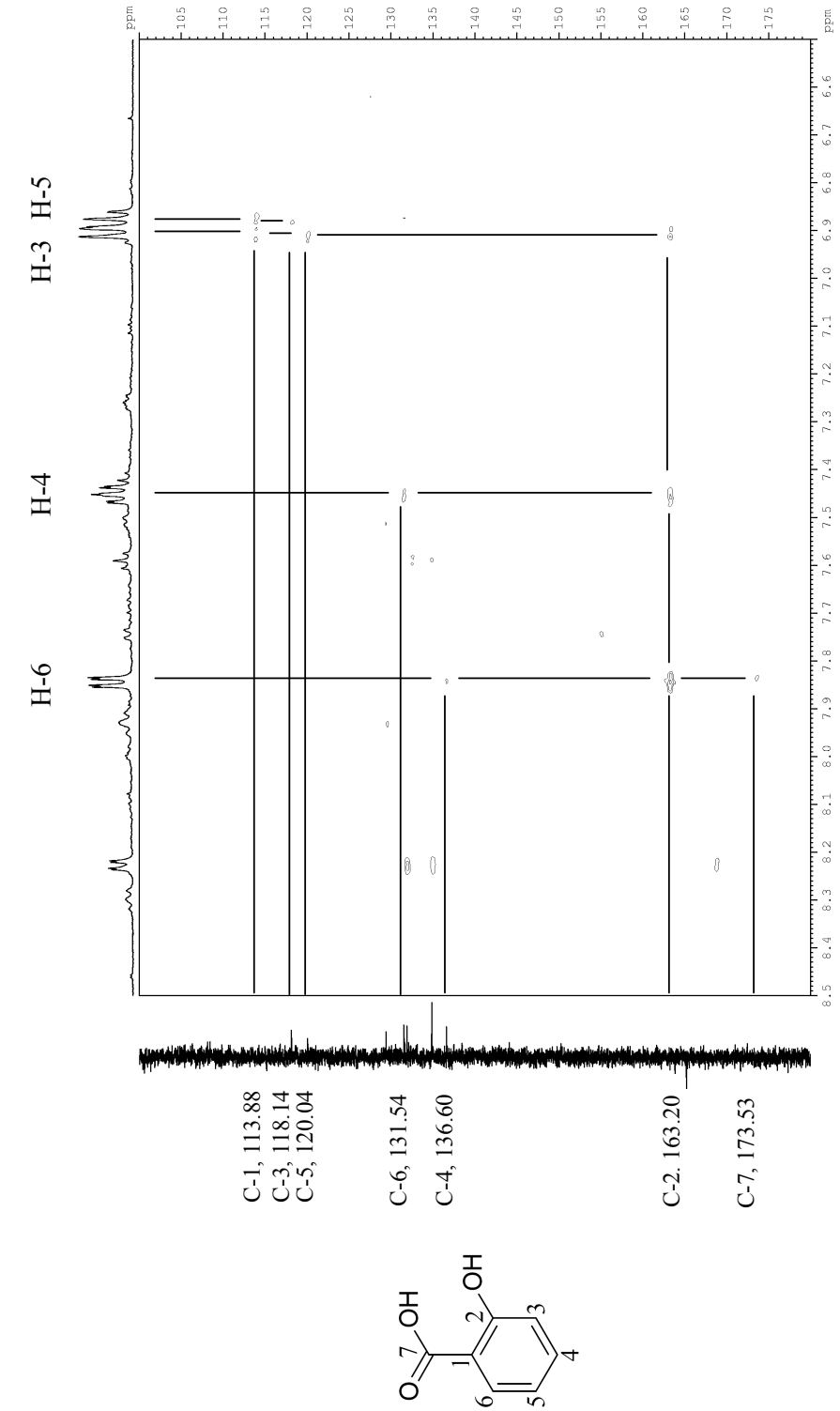

Figure 6.6. HMBC spectrum of purified extract of *Catharanthus roseus* elicited cells containing enriched SA (batch 1) dissolved in CH_3OH-d_4.

The SA signals in the ^{13}C-NMR spectra of the enriched samples were identified by comparison with those of the SA standard (Figure 6.4 C), assigned according to Scott (1972), and confirmed by the J-resolved ^{1}H-NMR spectra of the enriched SA batch 1 (Figure 6.5 A) and SA standard (Figure 6.5 B) and the HMBC spectrum of batch 1 (Figure 6.6).

Inverse gated decoupling was used in order to minimize the nuclear Overhauser effect (NOE), which occurs in general ^{13}C-NMR broadband decoupling. This method inserts a pulse delay after the acquisition period in order to reestablish equilibrium particularly for ^{13}C nuclei with long relaxation times (e.g. quaternary carbons), furthermore a high number of scans is required to build up the signal intensity for a ^{13}C-NMR quantitative analysis (Silverstein and Webster, 1998).

For the sample of batch 3 we used a different NMR spectrometer (500 MHz, instead of 600 MHz) with about two-fold higher number of scans (72192), but a high noise-level hampered a good quantitation, though the result obtained was in accordance with the results of the analysis of batch 1 SA, non-symmetrical labeling of C-2 and C-6 and low labeling of C-7.

The peak intensity was measured as peak height since this provided more reliable data. Subsequently, the relative intensity of the signals was determined as X = a'/b' for the enriched sample and as Y = a/b for the standard compound, of which a and b are the peak heights of carbon atom a and b respectively of the standard compound, whilst a' and b' are the peak heights of the carbon atom a and b of the enriched compound. Carbon atom b is a selected peak as standard to express the others in a relative value. In this case b is the C-7 peak height. Enrichment ratio is obtained by normalizing an X/Y with the lowest X/Y value. For SA these data are shown in Table 6.2 and 6.3.

Table 6.2. The chemical shifts and the peak heights of carbon signals of SA.

Salicylic acid (SA)							
Carbon no.	Chemical shift (ppm)			Peak height (mm)		Relative intensity to C-7	
	Scott (1972)	standard	Enriched sample	standard	Enriched sample	Standard (Y)	Enriched sample (X)
C-1	113.0	113.9	113.9	39	22	1.000	1.100
C-2	162.6	163.2	163.2	42.5	50	1.090	2.500
C-3	117.8	118.1	118.1	122	52	3.128	2.600
C-4	136.2	136.6	136.6	116.5	54.5	2.987	2.725
C-5	119.5	120.1	120.1	121	46	3.103	2.300
C-6	131.0	131.5	131.5	119	120	3.051	6.000
C-7	172.3	173.6	173.6	39	20	1.000	1.000

Table 6.3. The enrichment ratios of the carbons of enriched SA in the extracts of *Catharanthus roseus* suspension cells fed with [1-^{13}C]-D-glucose and elicited (24 h) by *Pythium* extract.

Salicylic acid (SA)		
Carbon no.	Ratio of relative intensities of enriched-SA/SA standard (X/Y)	Enrichment ratio
C-1	1.100	1.48
C-2	2.294	**3.09**
C-3	0.831	1.12
C-4	0.912	1.23
C-5	0.741	1.00
C-6	1.967	**2.65**
C-7	1.000	1.35

2,3-DHBA was extracted with EtOAc from the medium of batch 1 in acidic condition (pH 4). In the aromatic region of the ^{1}H-NMR- and ^{13}C-NMR spectra of this non-purified ethyl-acetate extract, 2,3-DHBA appeared as the major compound. The signals were compared with those of 2,3-DHBA standard (Figure 6.7 and 6.8 respectively). The carbon-signals were assigned as reported by Scott (1972) and the intensity was measured based on peak height (Table 6.4). The enrichment ratios of the 2,3-DHBA signals are shown in Table 6.5.

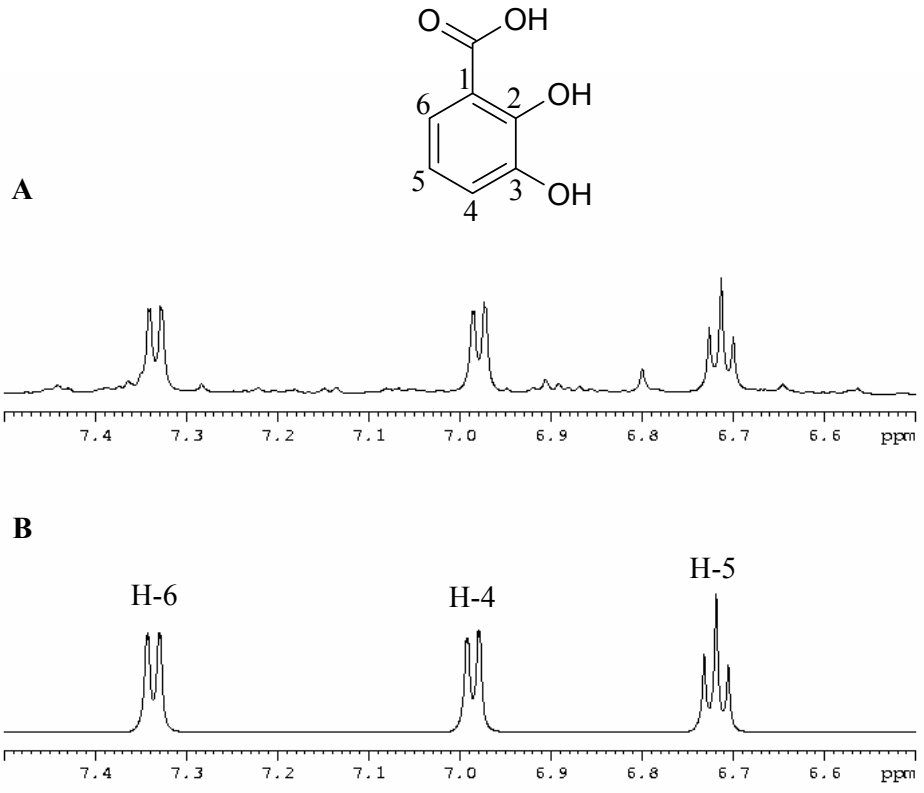

Figure 6.7. ^{1}H-NMR spectra (CH_3OH-d_4) of medium containing enriched 2,3-DHBA of batch 1 of *Catharanthus roseus* suspension cultures elicited by *Pythium* (A) and of 2,3-DHBA standard (B, 10 mg/mL).

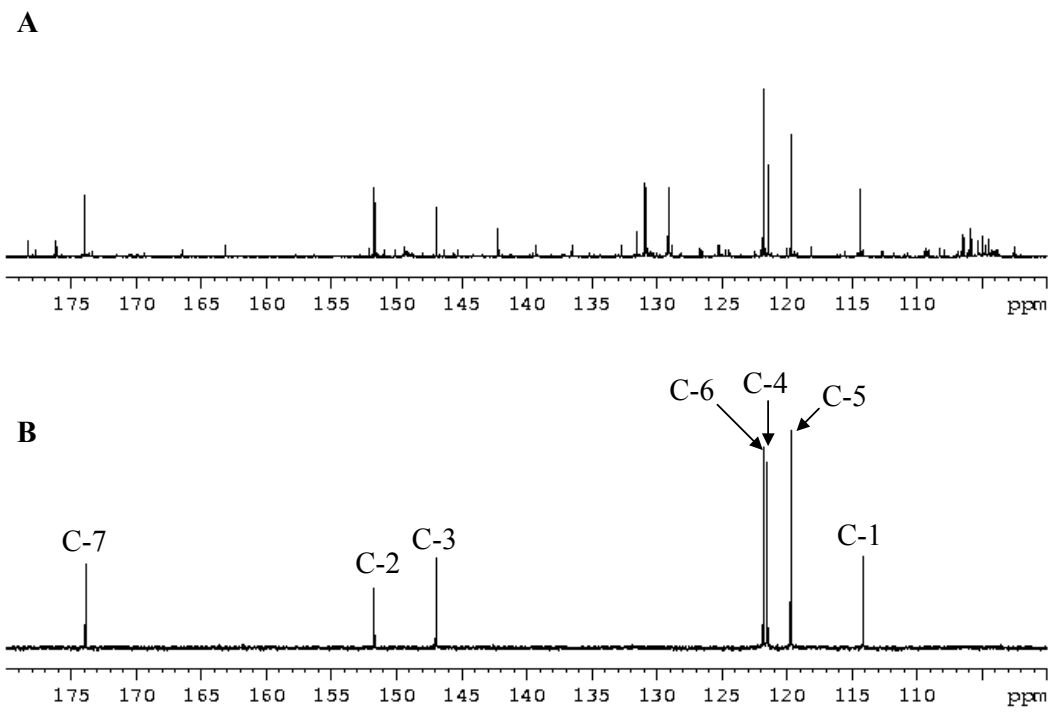

Figure 6.8. ^{13}C-NMR inverse gated spectra (CH_3OH-d_4) of medium containing enriched 2,3-DHBA of batch 1 of *Catharanthus roseus* suspension cultures elicited by *Pythium* (A) and -of 2,3-DHBA standard (B, 10 mg/mL).

The results show that relatively high- and non-symmetrical enrichment ratios occur at C-2 and C-6, and a lower enrichment ratio at C-7 of SA and 2,3-DHBA pointing to the isochorismate pathway as the responsible pathway for both compounds. In case of the phenylpropanoid pathway, the 2 and 6 positions become equivalent in phenylalanine resulting in a 1 : 1 ratio for labeling in those positions (Werner *et al*., 1997). In the isochorismate pathway those two carbons still are directly related to their respective precursors, and thus are likely to have different incorporation levels. Asymmetry of the labeling of C-2 and C-6 is thus evidence for the isochorismate pathway. The analyses of SA of the batch 3 spectra were in agreement with this conclusion.

Table 6.4. The chemical shifts and the peak heights of carbon signals of 2,3-DHBA.

2,3-dihydroxybenzoic acid (2,3-DHBA)							
Carbon no.	Chemical shift (ppm)			Peak height (mm)		Relative intensity to C-7	
	Scott (1972)	standard	Enriched sample	standard	Enriched sample	Standard (Y)	Enriched sample (X)
C-1	113.2	114.1	114.3	54	46	1.102	1.070
C-2	151.1	151.7	151.7	35	46.5	0.714	1.081
C-3	146.6	147.0	147.0	52	34	1.061	0.791
C-4	121.4	121.5	121.4	108	67	2.204	1.558
C-5	119.6	119.7	119.6	127	84	2.592	1.953
C-6	121.4	121.8	121.8	117.5	120	2.398	2.791
C-7	172.9	173.9	174.0	49	43	1.000	1.000

Table 6.5. The enrichment ratios of the carbons of enriched 2,3-DHBA in the extract of medium of *Catharanthus roseus* suspension cultures (batch 1) fed with [1-^{13}C]-D-glucose and elicited (24 h) by *Pythium* extract.

2,3-dihydroxybenzoic acid (2,3-DHBA)		
Carbon no.	Ratio of relative intensities of enriched-2,3 DHBA/2,3-DHBA standard (X/Y)	Enrichment ratio
C-1	0.971	1.37
C-2	1.514	**2.14**
C-3	0.745	1.05
C-4	0.707	1.00
C-5	0.753	1.07
C-6	1.164	**1.65**
C-7	1.000	1.41

The precursors of isochorismate are part of a large metabolic network, in which the label can be moved to almost any position. For example, both PEP and E4P can be synthesized from glyceraldehyde-3-P (3PGAL) through glycolysis/EMP pathway and the Pentose Phosphate - Entner-Doudoroff pathway (PP-ED pathway). Gluconeogenesis can move the label from C-1 to C-6 of glucose. Both different labeled glucoses can enter the oxidative- and/or non-oxidative PP-ED pathway resulting in labeled- and/or non-labeled 3PGAL and pyruvate. PEP alone can be produced from oxaloacetate through the Krebs cycle. The Krebs cycle/ the citric acid cycle is the second step in carbohydrate metabolism employing acetyl-CoA, which derives from pyruvate through glycolysis and PP-ED pathway (Figure 6.9). However, acetyl-CoA may also derive from amino acids through protein catabolism and/or from β-oxidation of fatty acids in fat catabolism. Depending on the activity of those pathways (the flux), the ratio of the differently-labeled precursors entering the shikimate pathway will be different through time. If SA and 2,3-DHBA are synthesized using the same isochorismate pool at the same time and place, both compounds are expected to have the same labeling pattern. Table 6.3 and 6.5 show that the highest incorporation occurred at C-2 and C-6 (of SA and 2,3-DHBA), which originate from [3-^{13}C]PEP and [4-^{13}C]E4P. However, the enrichment ratios of those carbons of SA are higher than of 2,3-DHBA. It seemed that glycolysis employing [1-^{13}C]glucose was probably the main pathway of the precursors for SA and as the time went on, scrambling of label occurred through other pathways e.g. gluconeogenesis, the Krebs cycle and liberation of glucose from non-labeled storage carbohydrates resulting in relative lower enrichment ratios of C-2 and C-6 of 2,3-DHBA. Many studies showed that an elicitation (a stress factor) increases the free-sugars levels in the cells. The metabolite profiling of SA-treated cells (Chapter 7 of this thesis) showed that the elicitation with SA results in an immediate increase of glucose levels, indicating to a major change in the fluxes through the primary metabolites pathways.

The label at C-1 of SA or 2,3-DHBA must originate from [2-^{13}C]PEP. This labeled-PEP can be formed through the Krebs cycle. [3-^{13}C]Pyruvate can be decarboxylated leading to carbon-dioxyde and [2-^{13}C]acetyl-CoA, the latter can be attached to oxaloacetate (entering the Krebs cycle) to produce citrate. In the Krebs cycle, the fumarase oxidizes fumarate into malate, subsequently malate dehydrogenase converts malate into oxaloacetate, the latter can be decarboxylated

resulting in [3-^{13}C]PEP and/or [2-^{13}C]PEP (the chance is 50% each) from only 1 turn of the cycle.

If the isochorismate pathway was the main pathway used for SA and 2,3-DHBA, the labeling at C-7 should be from [1-^{13}C]PEP, which can also be obtained from the Krebs cycle. The intermediates fumarate, malate and oxaloacetate will be labeled at the C-terminal side after the 3rd-time entering the Krebs cycle, thus producing [1-^{13}C]PEP. The chance of the labeled-carboxyl group remaining in PEP by decarboxylation of oxaloacetate is 50%, which is determined by the position of the keto group (as the result of the oxidation by fumarase) next to the labeled-carboxyl group. Thus, both C-1 and C-2 of PEP may be labeled. The labeling is likely to increase through time, with other words compounds formed directly from glucose after elicitation will have a relatively high level of incorporation at C-2, C-6 and in case of the phenylalanine pathway at C-7, whereas compounds formed later may have increased incorporation levels at C-1 and in case of isochorismate pathway at C-7, and thus the relative incorporation of all carbons C-1, C-2, C-6 and C-7 will be less.

The different labeling pattern of SA and 2,3-DHBA may be explained by the different time of the biosynthesis of both compounds after elicitation. In our previous studies (Budi Muljono, 2001) we found that SA biosynthesis started earlier than 2,3-DHBA after elicitation, probably because SA is a signal compound in SAR/ plant defense response among others resulting in the production of defense compounds such as 2,3-DHBA. Free SA and free 2,3-DHBA levels were always higher than the corresponding glucosides at every observation. Free SA was detected in a range of 0-325 ng/g fresh weight (FW) cells, whereas free 2,3-DHBA was found in a range of 0-200 μg/g FW cells (more or less 1000-fold higher than of SA level). The highest increase in SA level (from about 50 ng to 200 ng/g FW cells) occurred during 8 h after elicitation, whilst in the case of 2,3-DHBA this occurred at 20 h after elicitation (from about 70 μg to 150 μg/g FW cells). The 2,3-DHBA produced in elicited *C. roseus* cells was released into the medium. The pool of precursors from which most of 2,3-DHBA is produced is thus different than for SA. Besides the different time of biosynthesis, compartmentation on the level of cellular compartments or cells can not be excluded.

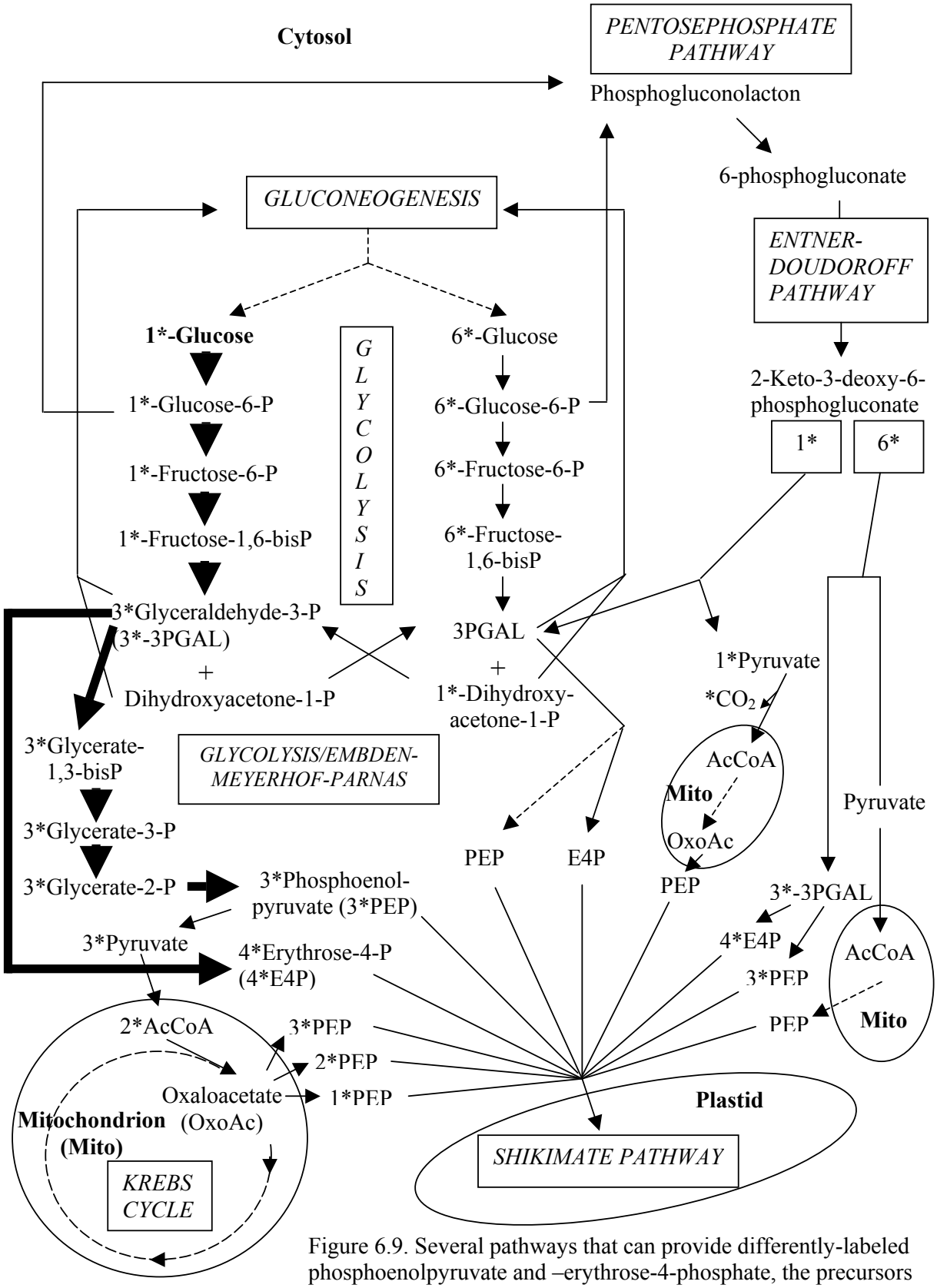

Figure 6.9. Several pathways that can provide differently-labeled phosphoenolpyruvate and –erythrose-4-phosphate, the precursors of chorismate/isochorismate. *: position of label

6.4 Conclusion

The labeling results found lead to the following conclusions: (1) The isochorismate pathway is responsible for the production of SA and 2,3-DHBA in *C. roseus* cell cultures elicited by *Pythium aphanidermatum* extract, (2) The different enrichment ratios of the carbons in SA and 2,3-DHBA mean that they are not produced from the same isochorismate pool, which means that their biosynthesis is separated in time and/or space.

Chapter 7

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

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Abstract

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

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

7.1 Introduction

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

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

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

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

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

7.2 Materials and methods

7.2.1 Chemicals

7.2.1.1 Chemicals used in suspension culture

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

7.2.1.2 Chemicals used in extraction and NMR analysis

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

7.2.2 Plant cell cultures

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

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

7.2.3 Elicitation and harvesting cells

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

7.2.4 Extraction

Fifty mg of freeze-dried cells of each flask were put in a 2 mL-micro tube. Seven hundred fifty μl of CH_3OH-d_4 and 750 μl of KH_2PO_4-d_2 buffer in D_2O (pH 6.0) were used as the extraction solvent. The mixture was vortexed (2,500 rpm, 1 min) using a Vibrofix VF1 electronic vortex (IKA, Staufen, Germany), followed by sonication (20 min) in an ultrasonic bath and centrifugation (13,000 rpm, 15 min) using a BHG HermLe Z 231 M centrifuge (B. HermLe, Gosheim, Germany). The supernatant was transferred into a 5 mm-NMR tube for NMR measurement.

7.2.5 NMR measurements

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

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

7.2.6 Data analysis

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

7.3 Results and discussion

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

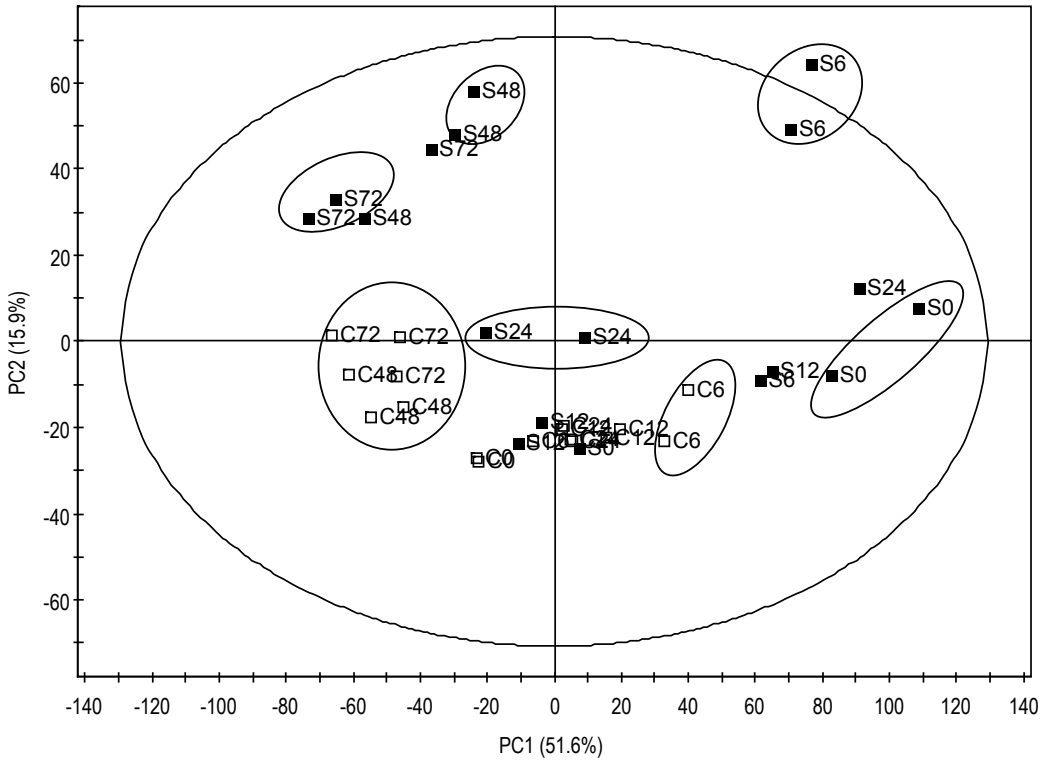

Figure 7.1. The score plot of PCA of ^{1}H-NMR data of *Catharanthus roseus* cells extracted with CH_3OH-d_4-KH_2PO_4 in H_2O-d_2 pH 6.0 (1 : 1). C: control cells, S: SA-treated cells, the number after the symbol shows the observation time (hour) after treatment.

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

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

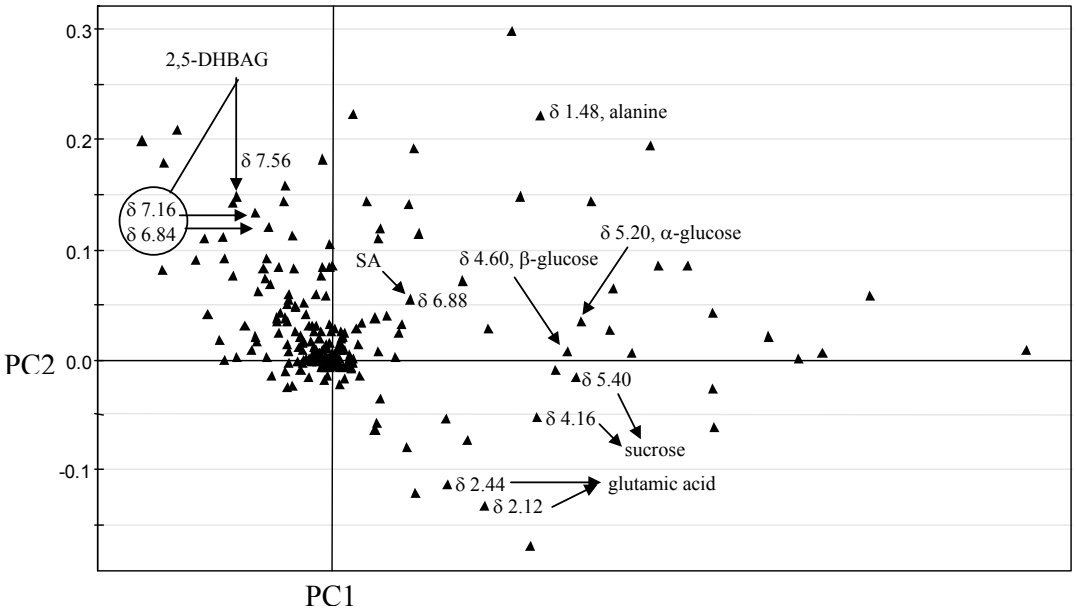

Figure 7.2. Loading plot of PC1 and PC2 of ^{1}H-NMR data of control- and SA-treated cells of *Catharanthus roseus*.

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

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

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

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

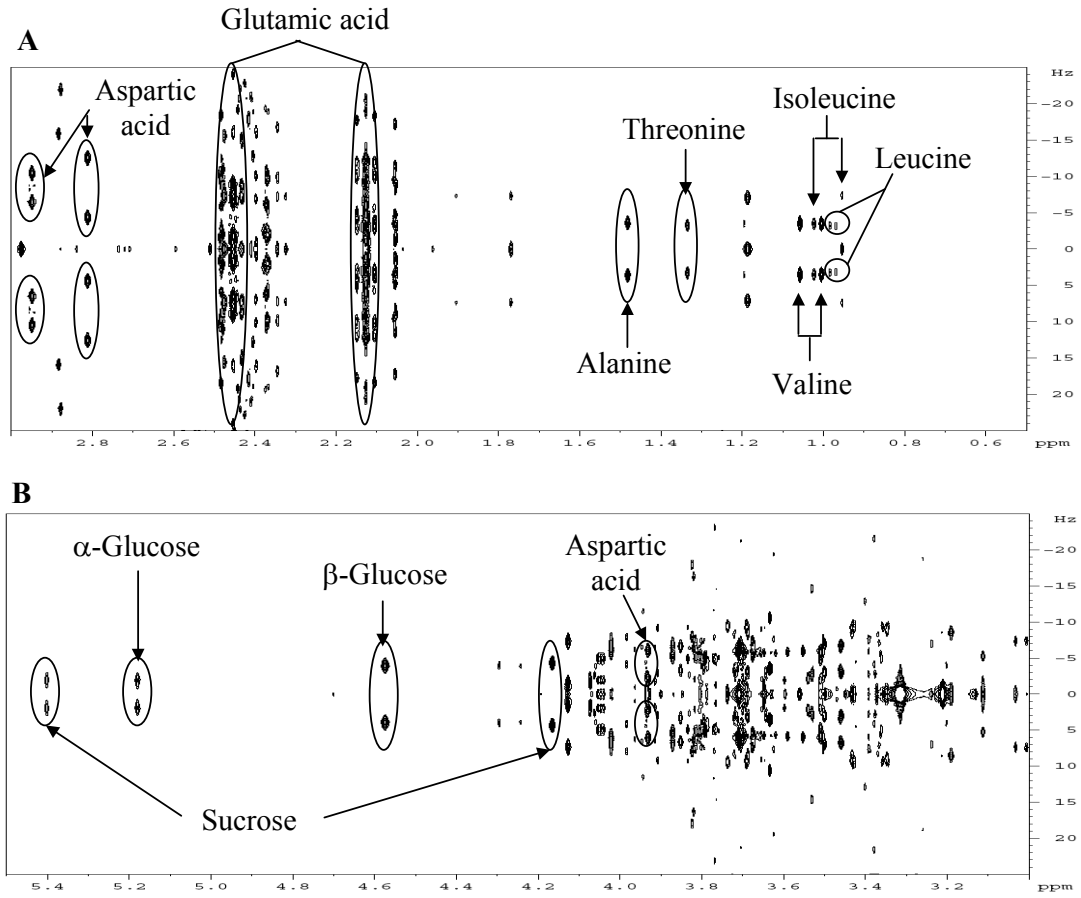

Figure 7.3. Some metabolites signals in the aliphatic region (A) and sugars region (B) of J-resolved ^{1}H-NMR spectrum (CH_3OH-d_4-KH_2PO_4 in H_2O-d_2 pH 6.0, 1 : 1) of the extract of *Catharanthus roseus* cells 12 h after elicitation with sodium-SA.

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

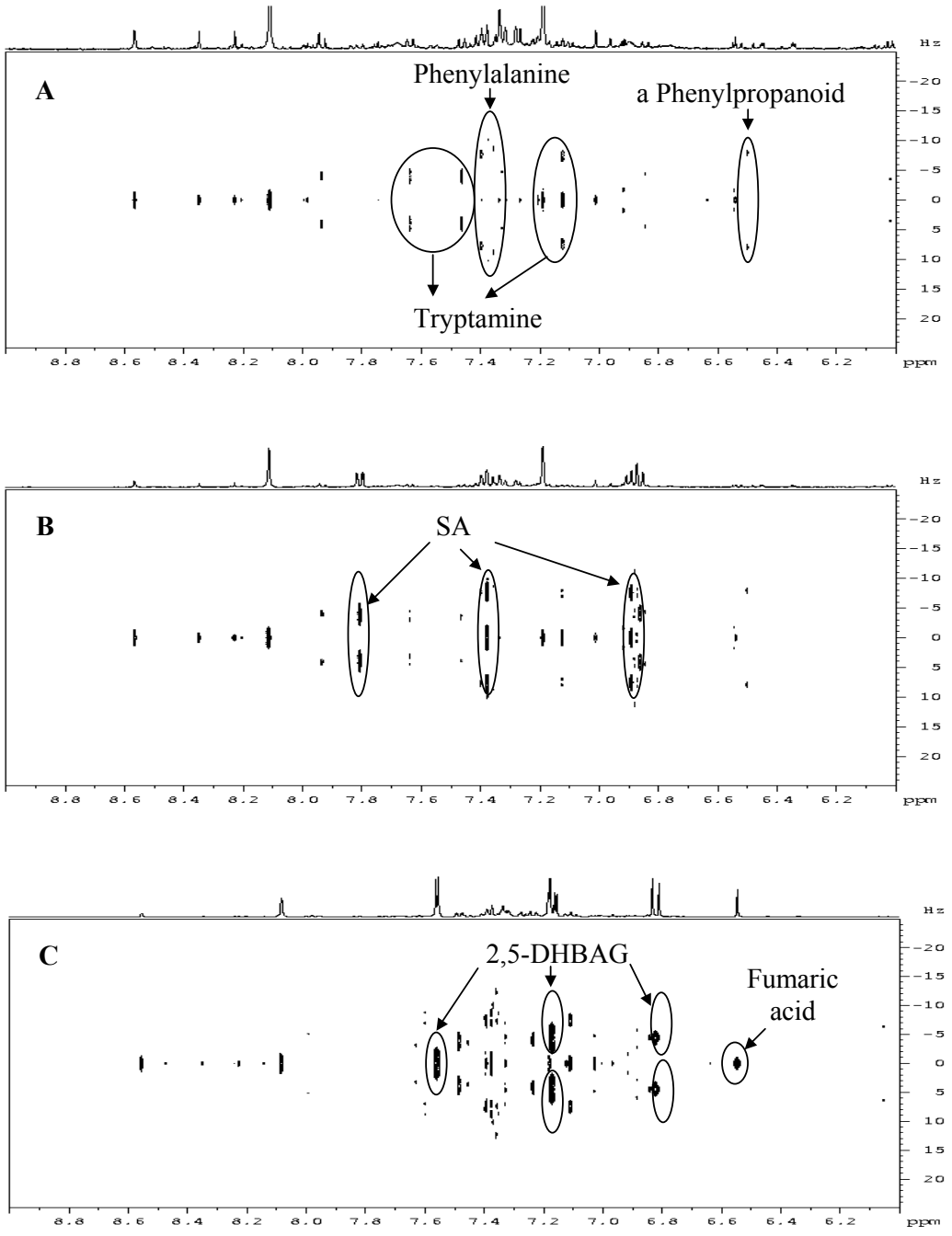

Figure 7.4. The signals of some compounds in the aromatic region of J-resolved ^{1}H-NMR spectra of *Catharanthus roseus* cells extracted with CH_3OH-d_4-KH_2PO_4 in H_2O-d_2 pH 6.0 (1 : 1). A: 6 h-control cells, B: 6 h-elicited cells, C: 48 h-elicited cells. SA= salicylic acid, 2,5-DHBAG= 2,5-dihydroxybenzoic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

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

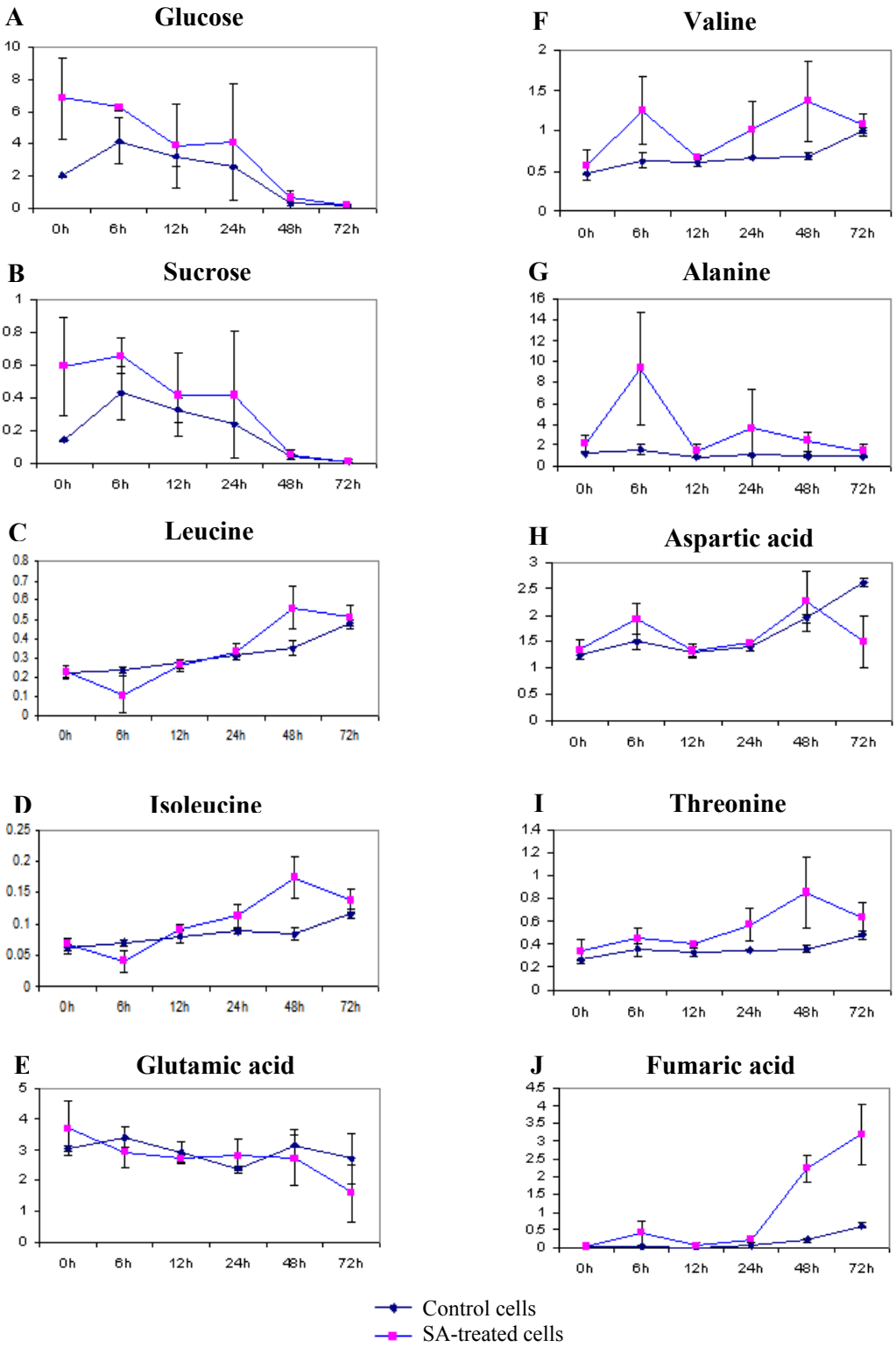

Figure 7.5. Time course (0-72 h) of the relative levels of sugars, some aliphatic amino acids and organic acids in the control- and SA-treated cells of *Catharanthus roseus* determined by a comparison of the peak-integrals of the compounds to the peak-integral of trimethylsilyl-propionate/ TSP (100%).

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

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

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

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

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

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

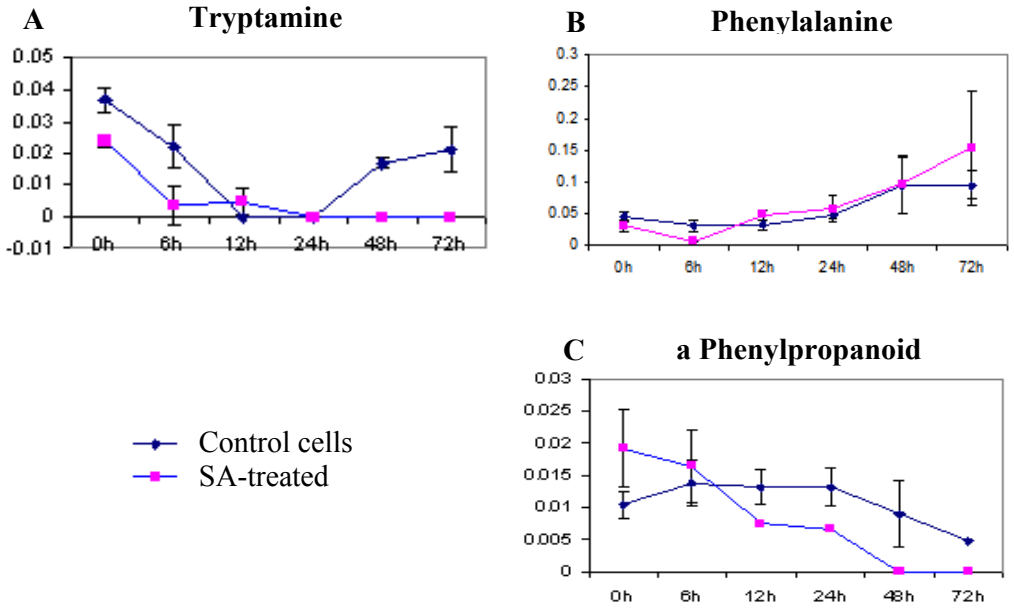

Figure 7.6. The levels of tryptamine (A), phenylalanine (B) and a phenylpropanoid (C) in the control- and SA-treated cells of *Catharanthus roseus* in a time course (0-72 h) determined by a comparison of the peak-integrals of the compounds to the peak-integral of TSP (100%).

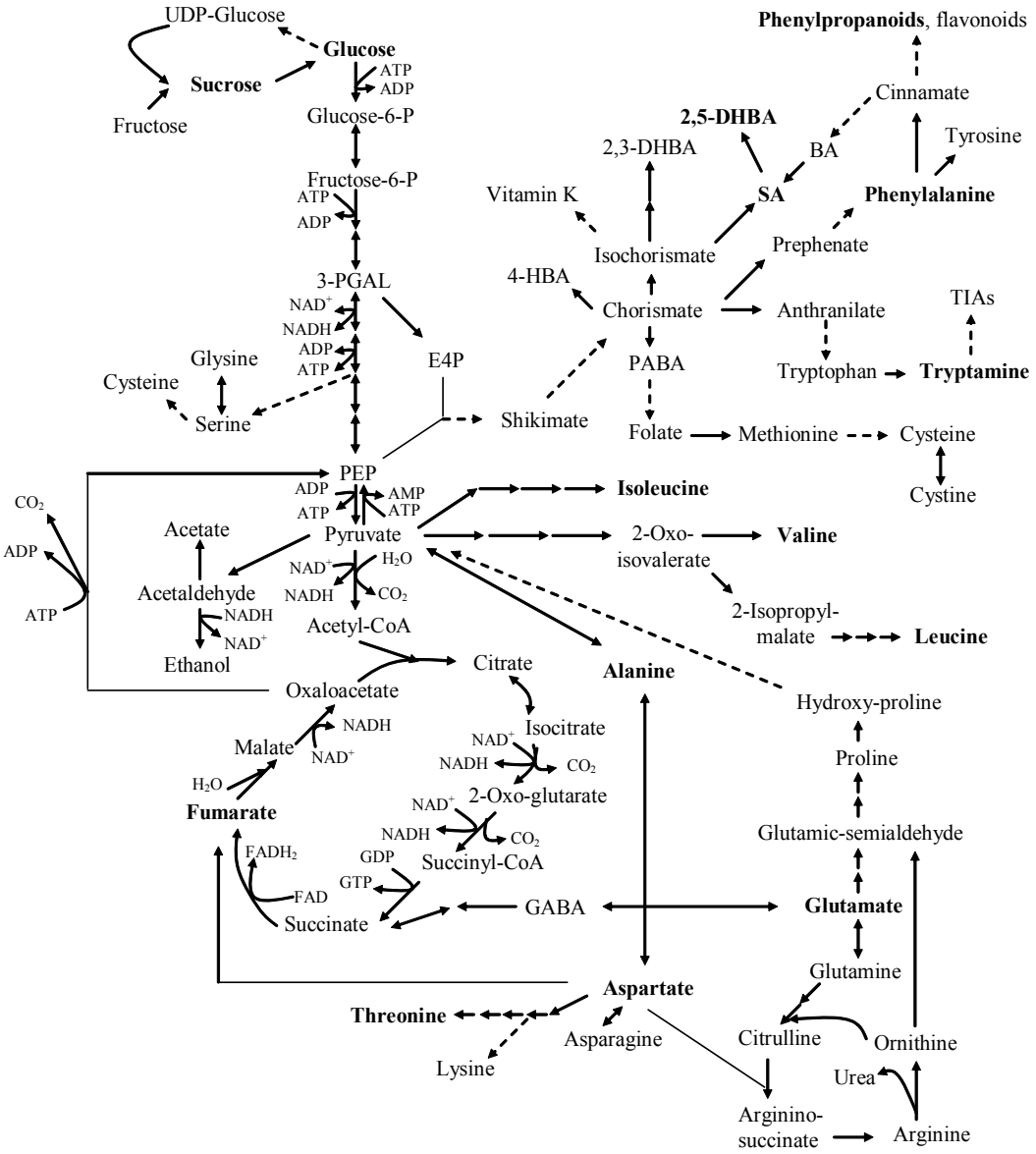

Figure 7.7. Glycolysis, The Krebs cycle and the biosynthetic pathways of some amino acids, organic acids, SA and 2,5-DHBA.

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

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

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

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

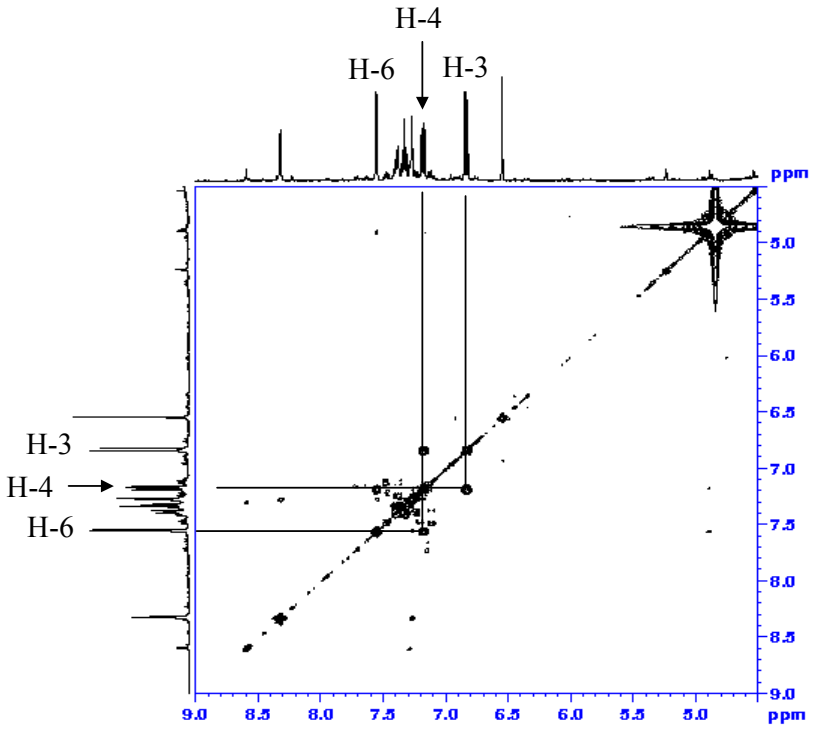

Figure 7.8. COSY spectrum (CH_3OH-d_4-KH_2PO_4 in H_2O-d_2 pH 6.0, 1 : 1) of the extract of *Catharanthus roseus* suspension cells 72 h after elicitation with sodium-salicylic acid, indicating the presence of 2,5-dihydroxybenzoic acid glucoside.

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

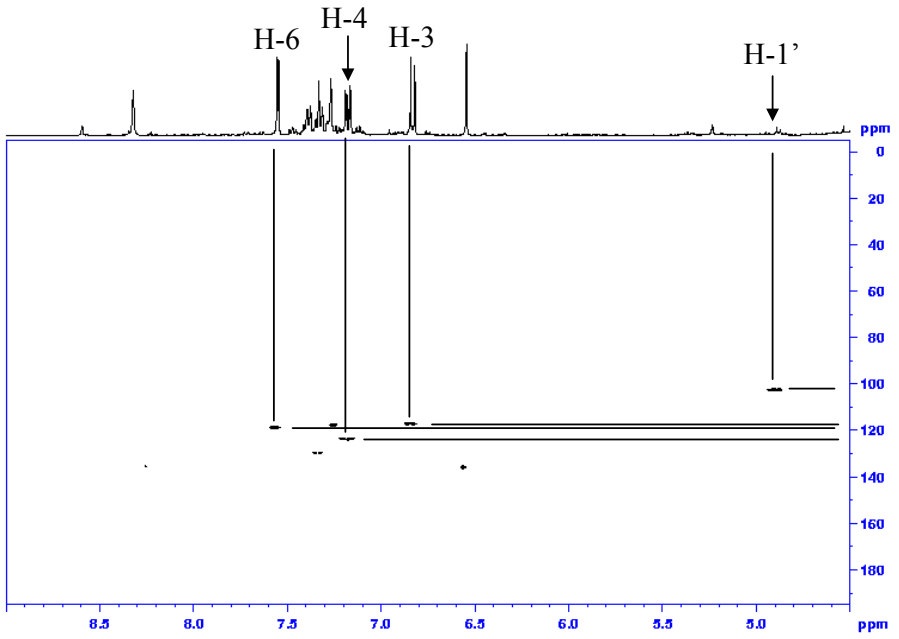

Figure 7.9. HMQC spectrum (CH_3OH-d_4-KH_2PO_4 in H_2O-d_2 pH 6.0, 1 : 1) of the extract of *Catharanthus roseus* suspension cells 72 h after elicitation with sodium-salicylic acid, indicating the presence of 2,5-dihydroxybenzoic acid glucoside.

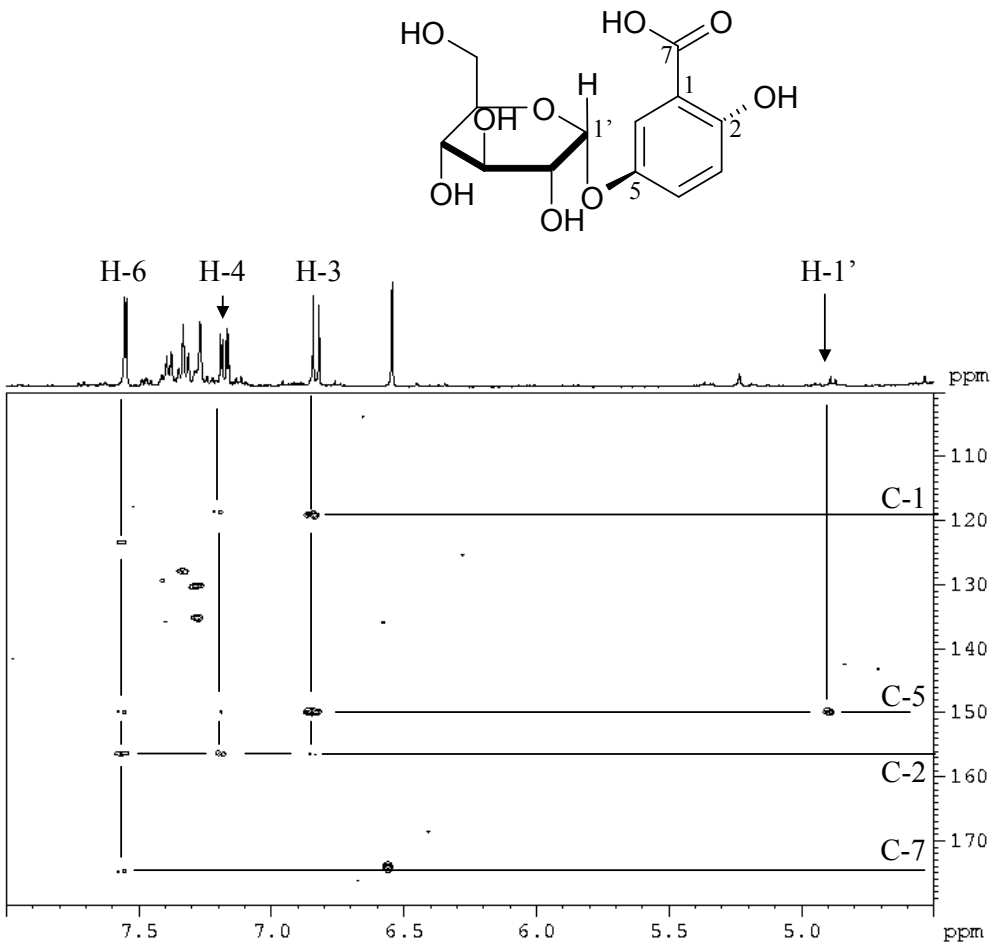

Figure 7.10. HMBC spectrum (CH_3OH-d_4-KH_2PO_4 in H_2O-d_2 pH 6.0, 1 : 1) of the extract of *Catharanthus roseus* suspension cells 72 h after elicitation with sodium-SA, indicating the presence of 2,5-dihydroxybenzoic acid glucoside (2,5-DHBAG).

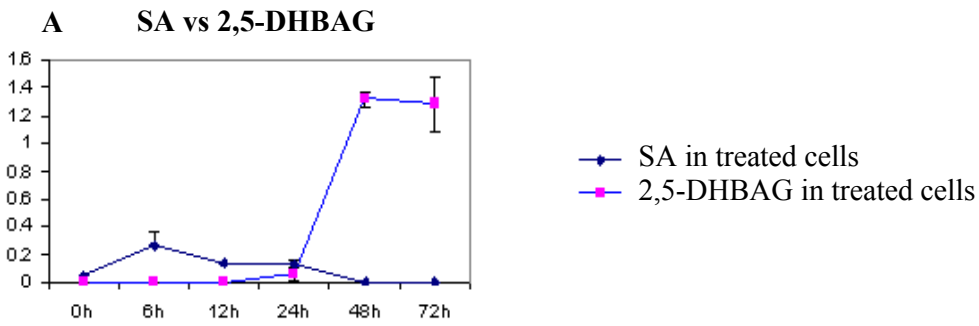

Figure 7.11. The levels of SA vs 2,5-DHBAG in the SA-treated cells of *Catharanthus roseus* in a time course (0-72 h) determined by a comparison of the peak-integrals of the compounds to the peak-integral of TSP (100%).

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

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

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

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

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

7.4 Conclusion

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

Summary

Plants produce a wide variety of secondary metabolites, which play a role in the interaction of plants with their environment. For example, they may act as attractants for pollinators, or as defense compounds against pathogens, insects, herbivores or abiotic stresses (e.g. UV-light, high-salinity and drought), or as signal compounds. Salicylic acid (SA) is a signal compound for so-called systemic acquired resistance (SAR). It belongs to C6C1 group of compounds, which structurally consists of a carboxyl group attached to an aromatic ring. SA derives from the shikimate pathway, a very important pathway in plants linking the carbohydrate metabolism to aromatic compounds metabolism. It consists of seven steps and it is the start for the biosynthesis of a broad spectrum of secondary metabolites e.g. phenolic compounds and alkaloids. The end product of the shikimate pathway is chorismate, which represents an important branching point, as it is a substrate of five different enzymes (reviewed in **Chapter 2**). The products of these branches are among others, phenylalanine, anthranilate, tryptophan, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid as well as SA. From the products a broad variety of secondary metabolites is derived. Unraveling the pathways leading to the C6C1 compounds in plants by isolation and characterization of the responsible enzymes and cDNAs are important not only for a basic understanding of SAR but also for application in metabolic engineering.

Phenolic compounds such as simple phenolics (C6), C6C1 compounds, simple phenylpropanoids (C6C3 compounds) and flavonoids (C6C3C6 compounds) are very common in plants and often increase in level upon a pathogenic attack. Also in *Catharanthus roseus* plants and cell cultures the presence of phenolic compounds was reported in several studies (reviewed in **Chapter 3**). *Catharanthus roseus* is a source of terpenoid indole alkaloids (TIAs) e.g. vincristine and vinblastine, the potent antitumor agents. In order to gain more insight in the flux through the pathway leading to desired metabolites like TIAs, studies about the biosynthetic pathways of other secondary metabolites and their regulation are necessary. The regulation of metabolic networks employs signal compounds such as SA, jasmonate (JA) and ethylene (ET). Different pathways can be activated depending on the plant environment or kind of biotic- or abiotic stress that a plant experiences. Differences in

the pathways activated, are not only found between species but also between plants, plant organs, cell cultures or seedlings of a species. For example elicitation of *C. roseus* cell cultures by *Pythium* extracts results in an increase of SA, 2,3-DHBA and tryptamine.

Since increased levels of SA and 2,3-DHBA (Budi Muljono, 2001) paralleled an increase in the isochorismate synthase (Budi Muljono *et al*., 2002) in *C. roseus* suspension cell culture upon elicitation by *Pythium* extract, the presence of the microbial pathway leading to SA in this species is plausible, as it was proposed by Verberne *et al*. (2000). Microorganisms convert chorismate to SA by isochorismate synthase (ICS) and isochorismate pyruvate-lyase (IPL), whereas SA in plants is thought to derive from phenylalanine, which involves many steps. Budi Muljono *et al*. (2002) proved the involvement of the microbial pathway for 2,3-DHBA by means of a retrobiosynthetic study. To proof this pathway also for SA, a high producing cell line is required because usually SA is produced in trace amounts only. Using a quantitative analysis HPLC method for SA developed by Verberne *et al*. (2002), we investigated the levels of SA upon elicitation with *Pythium* extract in some *C. roseus* cell lines for finding a high-SA-producing cell line necessary for labeling experiments (**Chapter 4**). We found that *C. roseus* grown in Murashige & Skoog medium without growth hormones (a wild-type A12A2 line) produced the highest level of total SA (free SA and SA-glucoside).

Besides the elicitation with *Pythium* for activation of the SA-pathway and for increasing the SA level, purification of SA is still necessary since the increased-level of SA still yields minor quantities as compared to other compounds, and consequently the signals of these compounds will interfer with SA-signals in the NMR spectra. We developed an anion exchange method to purify SA, consisting of a Dowex 1WX2 (100 mesh) as the resin, 0.25 mM sodium-phosphate pH (7.0-7.5) as the washing solvent and 0.3 M HCl in 60% of AcCN as the counter ion solution. It was observed that AcCN is better than MeOH as the organic solvent used in the counter ion solution. This system provides a good result as a single step purification of SA from plant cell cultures as observed in the (400 MHz) ^{1}H-NMR spectra (**Chapter 5**).

The availability of an efficient purification method for SA opened the possibility to perform the labeling studies. [1-^{13}C]-D-Glucose was used for the labeling experiment (**Chapter 6**). This labeled precursor is commonly used for retrobiosynthetic studies e.g. in yeasts and plants (Werner *et al*., 1997) including in *C.*

roseus cell cultures (Contin *et al*., 1998; Budi Muljono *et al*., 2002). Purification of a labeled crude extract of *C. roseus* elicited cells using the ion exchange chromatography system followed by a Sephadex-LH 20 column resulted in a well-purified enriched-SA extract as detected in the HMBC and ^{13}C-NMR spectra. The inverse gated ^{13}C-NMR method using a 600 MHz NMR spectrometer with a relatively high number of scans (36864) was used for the quantitative ^{13}C-NMR analysis of labeled-SA. The results showed a clear asymmetry of incorporation at C-2 and C-6, and a relatively low incorporation at C-7 of SA as well as of 2,3-DHBA, pointing to the isochorismate pathway as the responsible pathway for both SA and 2,3-DHBA in the *C. roseus* cell culture elicited by *Pythium* extract. The different patterns of label between SA and 2,3-DHBA shows that both compounds were synthesized at a different time and/or localization. Further investigations to phenylalanine derived compounds such as the phenylpropanoids found in our elicited cells, and an analysis of the metabolome of the cells should provide more insight in the pathways leading to other compounds derived from phenylalanine in *C. roseus*.

Metabolomics is a tool that should be able to give more information about possible metabolic changes after elicitation. By using NMR-based metabolomics, it is possible to get a general holistic view on all major compounds including both primary and secondary metabolites from a large variety of biosynthetic pathways (Kim *et al*., 2006). We applied this approach for following the effect of SA on *C. roseus* cells over a 72 h period (**Chapter 7**). We found that treatment of the *C. roseus* cells with 0.5 M sodium-SA changed the metabolites profile through time compared to non-treated cells. In the SA-treated cells, the highest levels of sugars were found at 0 h and these levels subsequently decreased to zero after 72 h. The changes in the levels of some aliphatic amino acids and organic acids were also observed. SA signals disappeared at 48 h but at the same time the signals of 2,5-dihydroxybenzoate glucoside (2,5-DHBAG) increased significantly. This might be a catabolic product of SA as previously reported by Shimoda *et al*. (2002). However, we could not find the signals of TIAs and also the TIA precursors loganic acid and secologanin due to the very low levels of the compounds or total absence. Detection of compounds present in low levels needs further investigation using other analytical methods for example HPLC-DAD and/or LC-MS/GC-MS. Metabolic profiling of *C. roseus* suspension cells elicited by jasmonate, *Pythium* extract or other elicitors would be necessary for further investigation about activation of different pathways by different elicitors.

It has been shown in this thesis that metabolic profiling by ^{1}H-NMR in combination with Principal Component Analysis can provide information about the metabolic changes in the cells in a time course after elicitation. However, for mapping a pathway the involvement of certain intermediates has to be confirmed by ^{13}C-NMR analysis of the labeled compounds after feeding labeled precursors.

Perspectives:

An efficient (routine) rational metabolic engineering in plants is impossible without knowing the structure of the plant metabolic network involved including the fluxes through the pathways (Ratcliffe and Shachar-Hill, 2005). The recent successful metabolic engineering of microorganisms is due to the more simple structure of their metabolic networks. However, most plant secondary metabolites cannot be produced using microorganisms. To be able to understand the structure of plant metabolic networks in all its complexity would be a breakthrough since complexity is found at all levels of biological organization. The demand to learn to understand the structure of metabolic networks is huge for not only to fulfill the human curiosity, but also for the potential important applications of such knowledge. The blueprint of the phenotype lies in the genes, however, the final phenotype is the result of the interaction of the organism with its environment, genomics and transcriptomics alone can not only explain the phenotype. These omics plus proteomics and metabolomics are necessary to achieve this goal though many studies showed that there is no direct parallel correlation between transcription, enzyme activity and the corresponding metabolites. Metabolic profiling and metabolic flux analysis (fluxomics) are important tools for unraveling the pathways part by part, including assessment of the performance of parts of the network. Tracer experiments using a stable isotope to measure fluxes through pathways have been quite extensively performed for measuring fluxes in primary metabolism. Even though the difficulties one has to face, for example the low levels of secondary metabolites, it is now the time to start to apply fluxomics on both primary and secondary metabolites in plants to realize the final goal; mapping the total plant metabolic network, including its regulation.

Samenvatting

Planten produceren een grote verscheidenheid aan secundaire metabolieten, die een rol spelen in de interactie tussen planten en hun omgeving. Zo kunnen deze stoffen bijvoorbeeld fungeren als aantrekkers van bestuivers, als een verdediging tegen pathogenen, insecten, herbivoren of abiotische stress (zoals UV-licht, hoge zout concentraties en droogte), of als signaalstoffen. Salicylzuur (SA) is een signaalstof voor de zogenoemde systemisch verkregen afweer (systemic aquired resistance, SAR). Het behoort tot de C6C1 klasse van stoffen, welke structureel zijn opgebouwd uit een carboxyl-groep (C1) gekoppeld aan een aromatische ring (C6). SA is afgeleid van de shikimaat pathway (biosynthese route), een in planten belangrijke pathway die het metabolisme van koolwaterstoffen verbindt met die van aromatische stoffen. Het bestaat uit zeven stappen, en staat aan het begin van de biosynthese van een breed spectrum van secundaire metabolieten, waaronder fenolische verbindingen en alkaloiden. Het eindproduct van de shikimaat pathway is chorismaat, een belangrijk vertakkingspunt van pathways aangezien chorismaat een substraat is voor 5 verschillende enzymen (overzicht in hoofdstuk 2). De producten van deze biosynthetische aftakkingen zijn onder andere: fenylalanine, anthranilaat, tryptofaan, p-aminobenzoëzuur, para-hydroxy-benzoëzuur, 2,3-dihydroxybenzoëzuur (2,3-DHBA), en SA. Van deze verbindingen is vervolgens een grote verscheidenheid aan secundaire metabolieten afgeleid. Het ontrafelen van pathways die leiden tot de vorming van C6C1 verbindingen in planten, door middel van isolatie en karakterisering van de verantwoordelijke enzymen en cDNA, is belangrijk zowel voor een basaal begrip van het verschijnsel SAR, als ook voor toepassing ervan in 'metabolic engineering'.

Fenolische stoffen zoals simpele fenolen (C6), C6-C1 verbindingen, simpele fenylpropanoiden (C6C3 verbindingen) en flavonoiden (C6C3C6 verbindingen) komen algemeen voor in planten en hun gehalte neemt vaak toe als gevolg van een microbiële infectie. Ook voor *Catharanthus roseus* planten en celcultures is de aanwezigheid van fenolische stoffen beschreven in diverse studies (overzicht in hoofdstuk 3). *Catharanthus roseus* is een bron van enkele belangrijke terpenoid indol alkaloïden (TIAs) waaronder de potente antitumor middelen, vincristine en vinblastine. Om meer inzicht te verkrijgen in het functioneren van de pathways die

leiden tot gewenste metabolieten zoals TIAs, zijn studies nodig naar de biosynthese van andere secundaire metabolieten en naar de regulatie hiervan. De regulering van biosynthesewegen geschiedt door middel van signaalstoffen zoals SA, jasmonaat (JA) en ethyleen (ET). Afhankelijk van de omgeving waarin de plant verkeert of de soort (a)biotische stress die hij ervaart, kunnen verschillende pathways aktief zijn of worden geactiveerd. Verschillen in geactiveerde pathways worden niet alleen waargenomen tussen soorten, maar ook tussen verschillende individuele planten, planten organen, celcultures en zaailingen van een enkele soort. Een voorbeeld hiervan is dat elicitatie van *C. roseus* celcultures met behulp van *Phytium* extract resulteert in de toename van het gehalte aan SA, 2,3-DHBA en tryptamine.

In *C. roseus* cel suspensie cultures gaan verhoogde gehaltes van SA en 2,3-DHBA (Budi Muljono, 2001) vergezeld met een toename in het enzym isochorismaat synthase (Budi Muljono *et al*., 2002) na elicitatie met *Pythium* extract. Dat maakt de aanwezigheid van de microbiële SA pathway in deze plant waarschijnlijk, zoals eerder voorgesteld is door Verberne *et al.* (2000). In micro-organismen wordt chorismaat omgezet in SA door isochorismaat synthase (ICS) en isochorismaat pyruvaat-lyase (IPL), terwijl gedacht wordt dat SA in planten afkomstig is van fenylalanine, waarvoor vele stappen nodig zijn. Budi Muljono *et al.* (2002) toonde de betrokkenheid van de microbiële pathway aan voor 2,3-DHBA door middel van een retrobiosynthetische studie. Om deze pathway ook aan te tonen voor SA is een hoog-producerende cellijn nodig, aangezien SA gewoonlijk slechts in zeer kleine hoeveelheden wordt geproduceerd. Met gebruikmaking van een kwantitatieve HPLC methode voor SA bepaling (Verberne *et al*., 2002) onderzochten we daarom de gehaltes aan SA in verschillende *C. roseus* cellijnen na elicitatie met *Pythium* extract, om een hoog-SA producerende cellijn te vinden die nodig is voor het verrichten van labeling-studies (hoofdstuk 4). We ontdekten dat een wildtype *C. roseus* cellijn (A12A2), gecultiveerd in Murashige & Skoog medium zonder groeihormonen, het hoogste gehalte produceerde aan totaal SA (som van vrije SA en SA-glucoside).

Uiteindelijke analyse van SA in de celcultures zal gebeuren met behulp van NMR technieken. Maar hiervoor is het verhogen van het SA gehalte door activeren van de SA pathway met behulp van *Pythium* extract niet voldoende, aangezien zelfs een verhoogd gehalte nog veel te laag is om te analyseren temidden van de andere celcomponenten, die zullen interfereren met de signalen van SA in de NMR-spectra. Daarom ontwikkelden we een methode voor de zuivering van SA met behulp van

anion-wisselings chromatografie, met Dowex 1WX2 (100 mesh) als stationaire fase, 0.25 mM natrium-fosfaat (pH 7.0-7.5) als wasbuffer en 0.3 M HCl in 60% acetonitril als counter-ion oplossing. Dit systeem resulteert in een goede eenstaps zuivering van SA uit plantencel cultures, zoals te zien is in de verkregen (400 MHz) ^{1}H-NMR-spectra (hoofdstuk 5).

De beschikbaarheid van een efficiënte zuiverings methode voor SA opende de mogelijkheid tot het verrichten van labeling studies met behulp van [1-^{13}C]-D-glucose (hoofdstuk 6). Deze gelabelde precursor wordt algemeen gebruikt voor retro-biosynthetische studies, zoals in gisten en planten (Werner *et al*., 1997), waaronder *C. roseus* celcultures (Contin *et al*., 1998; Budi Muljono *et al*., 2002). Zuivering van een gelabeld ruw extract van geëliciteerde *C. roseus* cellen met behulp van de ion exchange methode gevolgd door een Sephadex-LH20 kolom, resulteerde in een SA-verrijkt extract, zoals aangetoond in de verkregen HMBC- en ^{13}C-NMR spectra. Een 'inverse-gated' 13C-NMR methode, gebruik makend van een 600 MHz NMR-spectrometer en een relatief hoog aantal scans (36.864), werd gebruikt voor de kwantitatieve analyse van gelabeld SA. De resultaten tonen een duidelijke asymmetrie van incorporatie op posities C-2 en C-6, en een relatief lage incorporatie op C-7 van zowel SA als van 2,3-DHBA, wat aanduidt dat de isochorismaat pathway verantwoordelijk is voor de biosynthese van beide stoffen in *Pythium* geëliciteerde celcultures van *C. roseus*. De verschillen in de patronen van labeling tussen SA en 2,3-DHBA tonen dat beide stoffen geproduceerd zijn op verschillende tijdstippen en/of locaties in de celcultuur. Verdere studies naar stoffen afgeleid van fenylalanine, zoals de fenylpropanoiden aangetroffen in onze geëliciteerde cellen, en naar het metaboloom van deze cellen kan meer inzicht verschaffen in andere van fenylalanine afgeleide biosynthesewegen.

Metabolomics is een techniek die mogelijk informatie kan verschaffen over mogelijke metabolische veranderingen na elicitatie. Door middel van NMR-gebaseerde metabolomics is het mogelijk om een algemeen, holistisch overzicht te verkrijgen van alle hoofdcomponenten, waaronder zowel primaire als secundaire metabolieten afkomstig van een grote variatie aan biosynthetische pathways (Kim *et al*., 2006). We hebben deze methode toegepast bij het volgen van het effect van SA toediening op *C. roseus* cellen over een periode van 72 uur (hoofdstuk 7). Het bleek dat in deze periode het metabolieten profiel van deze cellen veranderde als gevolg van behandeling met 0.5 M natrium-SA, ten opzichte van niet-behandelde cellen. In de

SA-behandelde cellen werd het hoogste gehalte aan suikers gemeten bij aanvang (0 uur), waarna deze vervolgens afnamen tot ondetecteerbare gehaltes na 72 uur. Veranderingen in de gehaltes van bepaalde alifatische aminozuren en organische zuren werden ook waargenomen. NMR-signalen van SA verdwenen na 48 uur, maar gelijktijdig namen de signalen van 2,5-dihydroxybenzoaat-glucoside (2,5-DHBAG) significant toe. Dit is mogelijk een katabolisch product van SA, zoals eerder beschreven door Shimoda *et al.* (2002). Echter, signalen voor TIAs of hun precursors loganinezuur en secologanine werden niet waargenomen, wat duidt op zeer lage gehaltes dan wel volledige afwezigheid. De detectie van componenten die aanwezig zijn in zeer lage concentraties moeten verder onderzocht worden met behulp van gevoeligere analytische technieken als HPLC-DAD, LC-MS en/of GC-MS die spesifiek gericht zijn op de detectie van deze stoffen. Verder is 'metabolic profiling' van *C. roseus* suspensie celcultures, geëliciteerd met methyl-jasmonaat, *Pythium* extract of andere elicitoren, nodig voor een beter begrip van de activatie van verschillende pathways door diverse elicitoren.

In dit proefschrift is aangetoond dat 'metabolic profiling' met behulp van ^{1}H-NMR in combinatie met 'Principal Component Analysis' (PCA) informatie kan verschaffen over de metabolische veranderingen die plaatsvinden in de bestudeerde cellen in de tijdsperiode na elicitatie. Echter, voor het daadwerkelijk in kaart brengen van een pathway moet de betrokkenheid van bepaalde intermediairen bevestigd worden door ^{13}C-NMR analyse van gelabelde stoffen na toediening van gelabelde precursors.

Perspectieven:

Het verrichten van efficiente, rationele 'metabolic engineering' in planten is niet mogelijk zonder kennis van de structuur en regulatie van de betrokken metabole netwerken van de plant (Ratcliffe en Shachar-Hill, 2005). De recente successen in 'metabolic engineering' bij micro-organismen is dan ook te danken aan de veel eenvoudiger structuur van hun metabolisme. Maar helaas kunnen de meeste secundaire metabolieten niet worden geproduceerd door micro-organismen. Indien we in staat zouden zijn om de structuur van plant metabolisme in al zijn complexiteit te begrijpen, dan zou dat een heuse doorbraak zijn, aangezien een dergelijke complexiteit een rol speelt op alle niveaus van biologische organisatie. De drang om de structuur van metabole structuren te doorgronden is groot niet alleen ter

bevrediging van de menselijke nieuwsgierigheid, maar ook vanwege de enorme potentie voor toepassing van die kennis. De blauwdruk van het fenotype wordt grotendeels bepaald door de genen, maar het uiteindelijke fenotype is het resultaat van de interactie van het organisme met zijn omgeving. Hierdoor zullen genomics en transcriptomics alleen nooit in staat zijn het fenotype volledig te verklaren. Hoewel deze –omics technieken, samen met proteomics en metabolomics, nodig zijn om dit doel uiteindelijk te kunnen bereiken, hebben diverse studies aangetoond dat er geen directe parallele correlatie is tussen transcriptie, enzym activiteit, en corresponderende metabolieten. 'Metabolic profiling' en metabole flux analyse (fluxomics) zijn daarom belangrijke technieken voor het, stukje bij beetje, ontrafelen van pathways en het beoordelen van het belang van ieder gedeelte van de enorme netwerken van pathways. Tracer experimenten, waarbij gebruik wordt gemaakt van stabiele isotopen om de flux door pathways te bepalen, zijn veelvuldig uitgevoerd bij onderzoek naar het primaire metabolisme. Hoewel er behoorlijke obstakels zijn te overwinnen, zoals de zeer lage gehaltes van de meeste secundaire metabolieten, wordt het nu tijd om de technieken van de fluxomics toe te gaan passen op zowel het primaire als het secundaire metabolisme van planten. Wellicht bereiken we hiermee dan het uiteindelijke doel: het in kaart brengen van het gehele metabole netwerk van de plant, inclusief zijn regulatie.

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Curriculum Vitae

Natali Rianika Mustafa was born on December 25, 1964 in Palembang, Indonesia. After finishing the senior high school in 1983, she was awarded a direct placement at the Department of Pharmacy of the Bandung Institute of Technology (ITB). She obtained the first degree (S1) in Pharmacy in October 1988. She obtained a practising pharmacist certificate in September 1989 after following a one-year professional pharmacist education. From November 1989 until July 1991 she worked for Kimia Farma, a state-owned pharmaceutical company in Bandung at the Department of Research and Development. From August 1991 until September 1996, she stayed in The Netherlands (Almere) accompanying her husband who was doing his PhD research at the Dutch National Aerospace Laboratory. During this period, while caring for her baby and family, she used the opportunity to learn Dutch until she obtained a MAVO-certificate in Dutch and the NTT-2 certificate for Dutch as a second language. She returned to Indonesia in 1996 and joined the "Foundation Agenda 21" till June 1999. She returned to Holland in July 1999, and in the period from September 1999 till March 2001 she followed the MSc program in Biodiversity and Natural Products at the Department of Pharmacognosy, LACDR, Leiden University. In March 2001 she obtained the MSc diploma. She started the PhD research in April 2002 at the Department of Pharmacognosy, Section Metabolomics, Institute of Biology, Leiden University under supervision of Prof. Dr. Robert Verpoorte. Recently she continues to do research in Plant Metabolomics and Plant Ecology of Institute of Biology of Leiden University.

Publications

Mustafa NR, Verpoorte R (**2006**) Phenolic compounds in *Catharanthus roseus*. *Phytochem Rev* (accepted).

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Acknowledgements

Praise is due to almighty Allah SWT with His compassion and mercifulness to give me a good life and a chance to open my eyes for the beauty of life sciences in plants as I learned during this study.

The work that has led to this dissertation has been extensive but also exciting and enriching. This dissertation would not have been finished without the support, help and encouragement from several people.

Anneke gave the administrative support, Kim and Young gave many suggestions during my experiments, Cees and Fons assisted in data processing at the NMR department, Teus maintained the HPLC equipment, Arno translated the summary in Dutch, Alfi & Ibrahim were the experts for NMR-figures, Barbara and Yarek maintained my cell cultures when I was on a holiday, Sanimah, Hwan and Isvett assisted maintaining the lab HB 044 (the kitchen).

Also, I would like to mention several persons who assisted me during the early stage of my PhD study: Thea, who introduced me to work with plant cell cultures, Marianne who taught me to analyze SA from plant material and Karl for preparing media. I am grateful to Em and Erica, who were very kind and helpful persons in the lab to discuss with, and to Alberto who provided SAG. Thanks are due to my colleagues/friends for their support and friendship: Anja, Antok, Retno, Magdi, Tri, Okta, Lili, Ika, Moses, Yuana-Mark, Hetty-Chris, Jip-Richard, Winder, Michel, Choi, Philipos, Rudy, Miyuki, Ivon, Renee, Frank Van der Kooy, Annelies, Aida, Alba, Ali, Muhammad, Hasnain, Hien, Federica, Roman, Sytze, Nancy, Esther, Claudia, Lien, Ichwan, Aziz.

Last but not least, I thank my husband Bambang, my daughter Karina, my parents and my parents-in-law for their endless love and support, also to my sisters/sisters-inlaw and my brother/brothers-in-law for their kind attention and encouragement.