Biosynthesis of salicylic acid in fungus elicited Catharanthus roseus cells

Mustafa, N.R.; Kim, H.K.; Choi, Y.H.; Erkelens, C.; Lafeber, A.W.M.; Spijksma, G.K.; ... ; Verpoorte, R.

Phytochemistry 70 (2009) 532–539

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Biosynthesis of salicylic acid in fungus elicited *Catharanthus roseus* cells

Natali R. Mustafa [a], Hye Kyong Kim [a], Young Hae Choi [a], Cornelis Erkelens [b], Alfons W.M. Lefeber [b], Gerwin Spijksma [c], Robert van der Heijden [c], Robert Verpoorte [a,*]

[a] Division of Pharmacognosy, Section Metabolomics, Institute of Biology, Leiden University, Einsteinweg 55, Leiden, The Netherlands
[b] Division of NMR, Institute of Chemistry, Leiden University, Leiden, The Netherlands
[c] Division of Analytical Biosciences, Leiden-Amsterdam Center of Drug Research, Leiden University, Leiden, The Netherlands

ARTICLE INFO

Article history:
Received 21 May 2008
Received in revised form 26 November 2008
Available online 27 February 2009

Keywords:
Salicylic acid biosynthesis
2,3-Dihydroxybenzoic acid
Isochorismate pathway
Phenylalanine pathway
Catharanthus roseus cell suspension cultures

ABSTRACT

Feeding experiments using [1-^{13}C]-D-glucose to *Catharanthus roseus* (L.) G.Don cell suspension cultures followed by elicitation with *Pythium aphanidermatum* extract were 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. However, different enrichment ratios of the labeled carbons in SA and 2,3-DHBA indicate the use of different isochorismate pools, which means that their biosynthesis is separated in time and/or space.

1. Introduction

Salicylic acid (SA) is a precursor of siderophores (e.g. enterobactin and pyocheline) produced by microorganisms for uptake of Fe^{3+} in an environment with highly insoluble $Fe(OH)_3$ (Weinberg, 1978). In plants, one of the important functions of SA is as a signal compound for inducing systemic acquired resistance (SAR) (reviewed by Garcion and Métraux, 2006; Verberne et al., 1999). This C6C1 compound is synthesized from chorismate, the end product of the shikimate pathway and a substrate for five enzymes (reviewed by Mustafa and Verpoorte (2005)) leading to a diversity of secondary metabolites in plants, for example the terpenoid indole alkaloids (reviewed by van der Heijden et al. (2004)) and the other phenolics in *Catharanthus roseus* (reviewed by Mustafa and Verpoorte (2007)). Up to now there are two possible pathways known to lead to SA in living organisms. The first one is the isochorismate pathway, which converts chorismate into isochorismate by isochorismate synthase (ICS, EC 5.4.99.6) followed by the conversion of isochorismate into SA by isochorismate pyruvate-lyase (IPL). The second pathway for SA is the phenylalanine pathway, which long has been 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)).

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 (Budi Muljono, 2001) 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., 1994; Budi Muljono et al., 2002). A retrobiosynthetic study with *C. roseus* suspension cells fed with [1-^{13}C]-D-glucose has shown the intermediacy of isochorismate in 2,3-DHBA biosynthesis rather

* Corresponding author. Tel.: +31 71 527 4528; fax: +31 71 527 4511.
E-mail address: verpoort@chem.leidenuniv.nl (R. Verpoorte).

0031-9422/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2009.01.009

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]-D-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. The [1-^{13}C]-D-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 Fig. 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]-D-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 Fig. 1. 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.

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 (Fig. 1). 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.

2. Results and discussion

Among four different *C. roseus* cell lines, the A12A2 cell suspension culture grown in Murashige and Skoog (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 (Mustafa, 2007). The cells were grown in M&S medium with a final concentration of 1% [1-^{13}C]-D-glucose w/v in the medium. This level of the 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. The attempt to reduce possible dilution is strongly necessary since the experiment deals with a plant hormone (signaling compound), which is produced by plant cells in low amounts (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). Aqueous methanol (90%, v/v) was used to extract the dried material and the purification of SA from the extracts was performed using the method previously described by Verberne et al. (2002) subsequently followed by an ion exchange chromatography (IEC) method described by Mustafa et al. (2008). The levels of free SA were determined by HPLC (Verberne et al., 2002) in the MeOH–water extracts (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 1). The determination of the free SA level of batch 3 before IEC was carried out in the MeOH–water extract collected from 48 h maceration (total 165 μg). By continuing maceration with a fresh volume of MeOH 90% v/v until 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 purification using IEC we could detect the SA resonances quite well among the signals of some remaining impurities (e.g. 2,3-DHBA). However, in the 2D 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 compounds with almost similar molecular weight (e.g. different in one hydroxyl group) can result in different retention. Besides, this method provides a good recovery (almost 100%). 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. The sample was measured twice using this method, and it was also measured once using broadband decoupling. All gave similar results. Fig. 2A shows the inverse gated ^{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 (Fig. 2B), however, the noise-level was higher than that of the batch 1 and therefore was only used for a semi-quantitative analysis (see below).

The SA signals in the ^{13}C NMR spectra of the enriched samples were identified by comparison with those of the SA reference compound (Fig. 2C), assigned according to Scott (1972), and confirmed by the J-resolved ^{1}H NMR spectra of the enriched SA batch 1 (supplementary figure 1A) and SA standard (supplementary figure 1B) and the HMBC spectrum of batch 1 (supplementary figure 2). The inverse gated decoupling spectral data was used for the

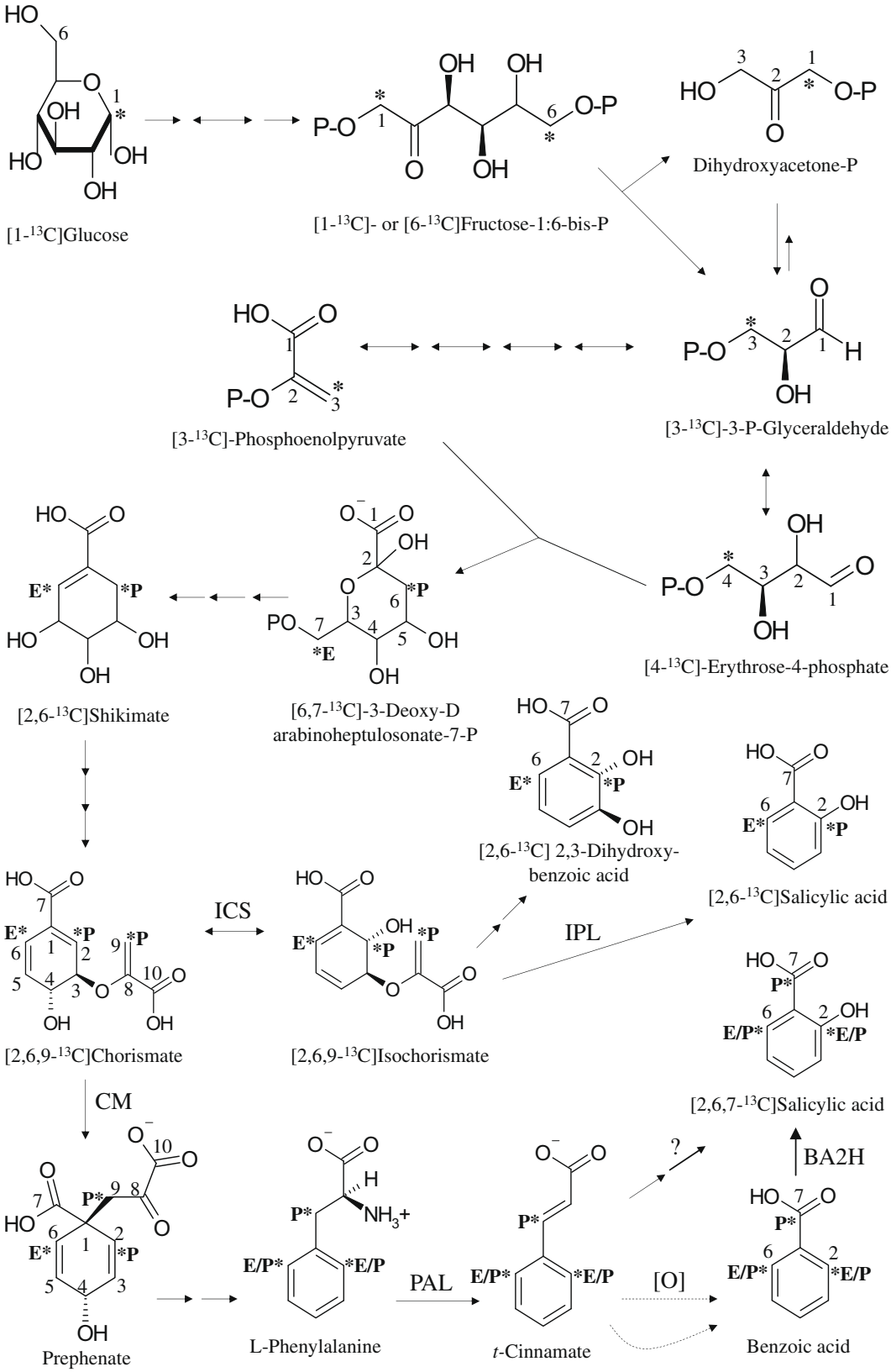

Fig. 1. The salicylic acid and 2,3-dihydroxybenzoic pathway. CM, chorismate mutase; ICS, isochorismate synthase; IPL, isochorismate pyruvate-lyase; PAL, phenylalanine ammonia-lyase; BA2H, benzoic acid 2-hydroxylase; [O], oxidative pathway; *E, label from erythrose-4-phosphate; *P, label from phosphoenol-pyruvate.

quantization of ^{13}C-signals (see Table 2 and 3). 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. For the sample of batch 3 we used a different NMR

Table 1
Amounts of salicylic acid harvested.

Batch number	g DW cells	% to FW	Amounts of free SA (μg)				
			Before IEC	After IEC		After fractionation by Sephadex LH-20[a]	
				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	×	×
3	9.53	4.2	>165	17	190	85	49

DW, dry weight; FW, fresh weight; IEC, ion exchange chromatography; *x*, not performed.
[a] For batch 1: free SA and SA after acid-hydrolysis were pooled and loaded on the column.

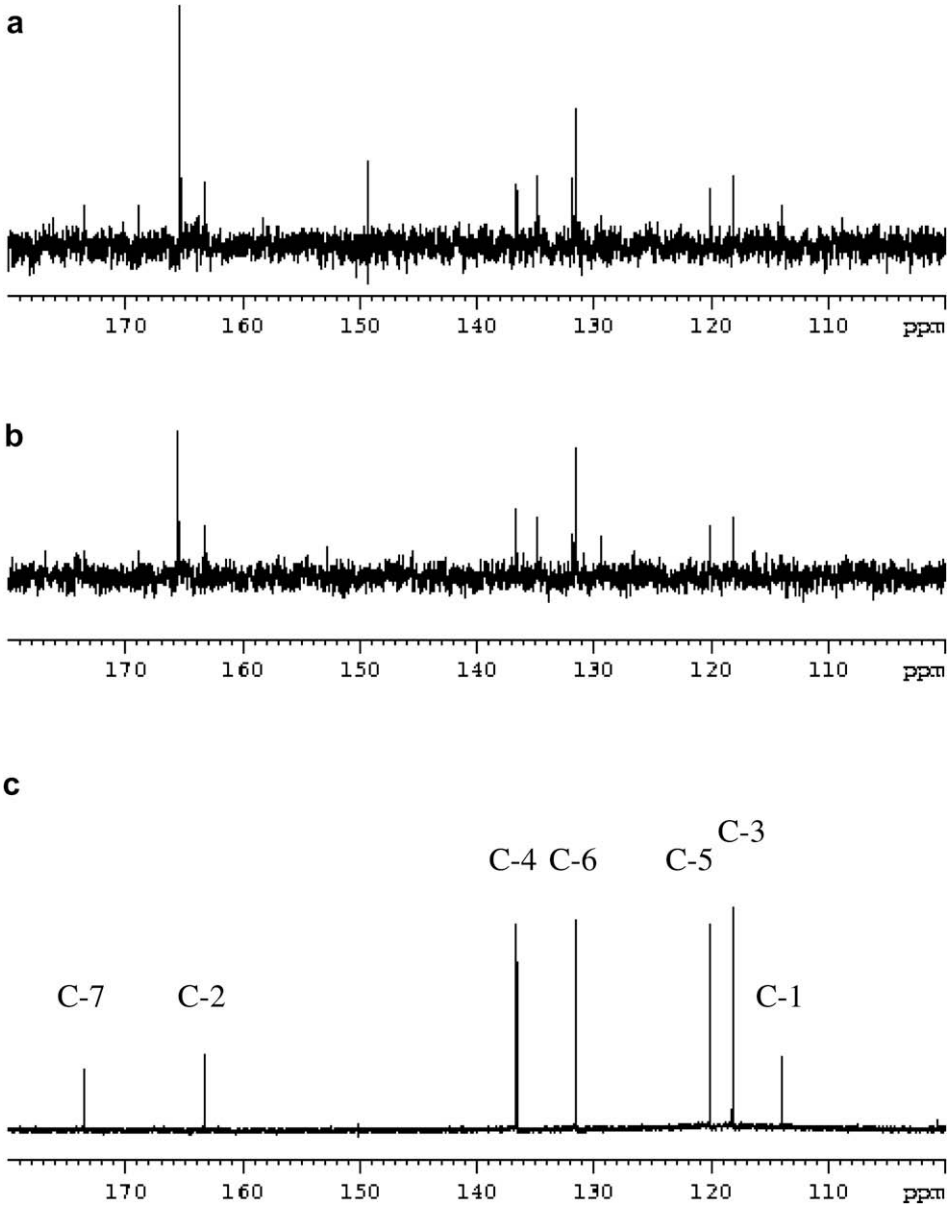

Fig. 2. ^{13}C NMR inverse gated spectra (CH_3OH-d_4) of purified extracts of *Catharanthus roseus* elicited cells containing enriched salicylic acid (a, batch 1 and b, batch 3) and of salicylic acid reference compound (c, 10 mg/ml).

spectrometer (500 MHz, instead of 600 MHz) with about 2-fold higher number of scans (72,192), but a high noise-level hampered an accurate quantization, though the semi-quantitative 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

Table 2
The enrichment ratios of the carbons of enriched salicylic acid (SA) in the purified extracts of fungal elicited *Catharanthus roseus* cells fed with [1-^{13}C]-D-glucose.

Carbon no.	Chemical shift (ppm)			Peak height (mm)		Relative intensity to C-7		*X*/*Y*	Enrichment ratio	Abundance (%)[a]
	Scott (1972)	SA standard	Enriched sample	SA standard	Enriched sample	SA standard (*Y*)	Enriched sample (*X*)			
C-1	113.0	113.9	113.9	39	22	1.000	1.100	1.100	1.48	3.58
C-2	162.6	163.2	163.2	42.5	50	1.090	2.500	2.294	**3.09**	**7.47**
C-3	117.8	118.1	118.1	122	52	3.128	2.600	0.831	1.12	2.71
C-4	136.2	136.6	136.6	116.5	54.5	2.987	2.725	0.912	1.23	2.97
C-5	119.5	120.1	120.1	121	46	3.103	2.300	0.741	1.00	2.42
C-6	131.0	131.5	131.5	119	120	3.051	6.000	1.967	**2.65**	**6.41**
C-7	172.3	173.6	173.6	39	20	1.000	1.000	1.000	1.35	3.26

[a] The absolute overall labeling percentage is determined by calculating the total of enriched carbons in the molecules from the peak ratios of $[M+1]^+$, $[M+2]^+$, $[M+3]^+$ and $[M+4]^+$ (data from LC–MS analysis, see supplementary figure 5).

Table 3
The enrichment ratios of the carbons of enriched 2,3-dihydroxybenzoic acid (2,3-DHBA) in the extracts of medium of fungal elicited *Catharanthus roseus* cells fed with [1-^{13}C]-D-glucose.

Carbon no.	Chemical shift (ppm)			Peak height (mm)		Relative intensity to C-7		*X*/*Y*	Enrichment ratio	Abundance (%)[a]
	Scott (1972)	2,3-DHBA standard	Enriched sample	2,3-DHBA standard	Enriched sample	2,3-DHBA standard (*Y*)	Enriched sample (*X*)			
C-1	113.2	114.1	114.3	54	46	1.102	1.070	0.971	1.37	2.44
C-2	151.1	151.7	151.7	35	46.5	0.714	1.081	1.514	**2.14**	**3.82**
C-3	146.6	147.0	147.0	52	34	1.061	0.791	0.745	1.05	1.87
C-4	121.4	121.5	121.4	108	67	2.204	1.558	0.707	1.00	1.78
C-5	119.6	119.7	119.6	127	84	2.592	1.953	0.753	1.07	1.91
C-6	121.4	121.8	121.8	117.5	120	2.398	2.791	1.164	**1.65**	**2.94**
C-7	172.9	173.9	174.0	49	43	1.000	1.000	1.000	1.41	2.51

[a] The absolute overall labeling percentage is determined by calculating the total of enriched carbons in the molecules from the peak ratios of $[M+1]^+$, $[M+2]^+$, $[M+3]^+$ and $[M+4]^+$ (data from LC–MS analysis, see supplementary figure 5).

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 2.

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 (supplementary figure 3 & Fig. 3). The carbon-signals were assigned as reported by Scott (1972) and the intensity was measured based on peak height. The enrichment ratios of the 2,3-DHBA signals are shown in Table 3. 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.

The precursors of isochorismate are part of a large metabolic network, in which the label can be moved to almost any position (as an illustration, see supplementary figure 4). 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 is the second step in carbohydrate metabolism employing acetyl-CoA, which derives from pyruvate through glycolysis and PP–ED pathway. 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. Tables 2 and 3 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 (Mustafa, 2007) 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 carbondioxyde 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 one 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 third-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.

A high resolution mass spectrometry analysis was also applied on the purified enriched SA extract- and enriched 2,3-DHBA extract of batch 1 (supplementary figure 5). The result shows that the $[M+2]^+$ fractions of both extracts were higher than those of the standards. All of the labeled fractions detected {$[M+1]^+$ until $[M+4]^+$} of enriched SA were in higher levels than those of enriched 2,3-DHBA. These results support the NMR results (the enrichment ratios of carbon atoms of SA and 2,3-DHBA), suggesting that dilution of label occurred more intensively at the time when 2,3-DHBA was synthesized than that of SA. Using the enrichment ratio of each carbon as determined by ^{13}C NMR and the total overall percentage of labeled carbon from ratio $[M+1]^+$ until $[M+4]^+$ the abundance (%) of label at each carbon position of SA and 2,3-DHBA can be calculated (Tables 2 and 3).

The different labeling pattern of SA and 2,3-DHBA and the results of the mass spectrometry analysis may also explain the different levels of the compounds in a time course 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 4–8 h after elicitation, whilst in the case of 2,3-DHBA this occurred at 16–20 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.

3. 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. In fact we are dealing with the four dimensions of a dynamic biological system. To elucidate the regulation we need at least to measure more points in time to be able to get insight in the fluxes through the pathways. The three dimensions of space, i.e. the compartmentation, are much more difficult to deal with as it requires separation of cell types and/or cell organelles, followed by analysis sufficiently sensitive to measure the compounds of interest. Developing fluxomics will thus be the major challenge.

4. Experimental

4.1. Plant cell cultures

Catharanthus roseus cell suspension cultures line A12A2 was provided from Delft University in 1995. Maintenance has been performed weekly and the cell line was also cryopreserved in our laboratory. This cell line was grown previously in Murashige and Skoog (M&S) medium (Murashige and Skoog, 1962) containing 3% sucrose, but then adapted in M&S medium without growth hormones supplemented with 2% of D(+)-monohydrate glucose as the carbon source for labeling purpose. The yellowish green A12A2 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.

4.2. 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. All experiments were performed with the same batch of filtrate.

4.3. Elicitation and harvesting cells

About 20 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 90%.

4.4. 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, $MnSO_4 \cdot H_2O$, Na_2EDTA, $ZnSO_4 \cdot 7H_2O$ (Merck) and $FeSO_4 \cdot 7H_2O$ (Brocades-ACF groothandel NV, Maarssen, The Netherlands) were dissolved in one solution, whereas $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, KI and $NaMoO_4 \cdot 2H_2O$ (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).

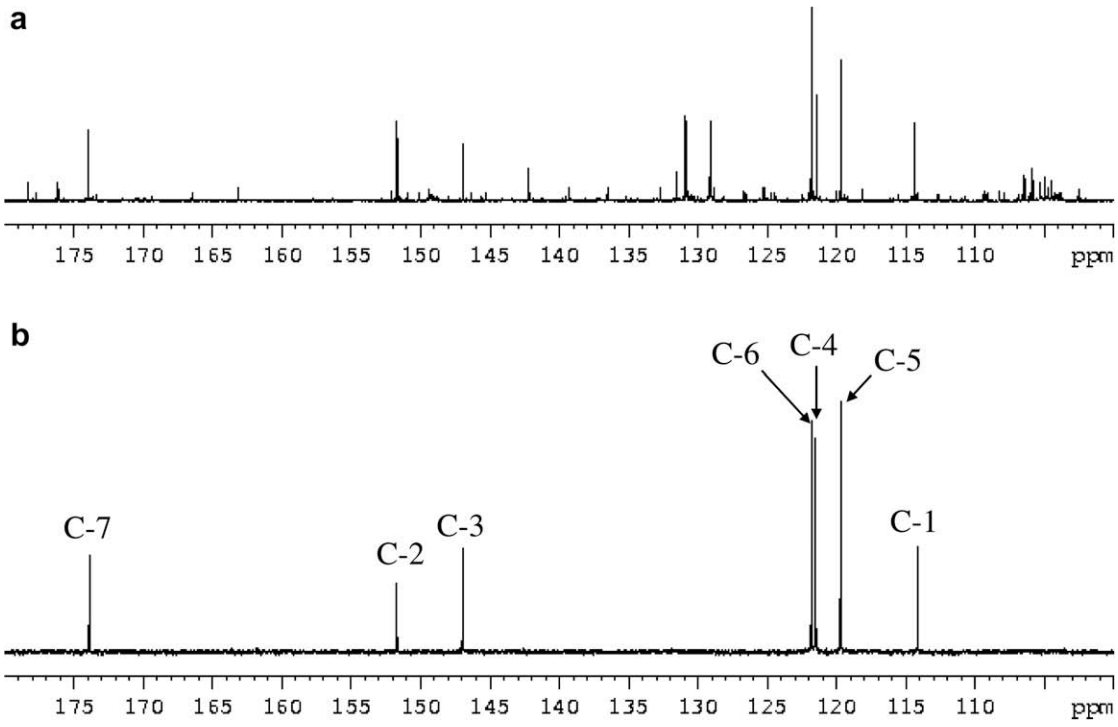

Fig. 3. ^{13}C NMR inverse gated spectra (CH_3OH-d_4) of medium containing enriched 2,3-dihydroxybenzoic acid of batch 1 of *Catharanthus roseus* suspension cultures elicited by *Pythium* (a) and of 2,3-dihydroxybenzoic acid reference compound (b, 10 mg/ml).

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).

4.5. 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).

4.6. 2,3-DHBA extraction

The method previously described by Budi Muljono et al. (2002) was used for 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.

4.7. Salicylic acid extraction

Twenty-four hours-elicited cells of *C. roseus* A12A2, harvested from 9 or 10 Erlenmeyer-flasks (each containing 100 ml) of cell suspension culture, were transferred to the same number of 50 ml-tubes and freeze–dried for 72 h. About 25 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 °C, 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–water extracts were pooled and the extraction of SA was performed using the method described by Verberne et al. (2002). One ml of 0.2 N NaOH was added into the MeOH–water extract and the solvent was evaporated to almost dry, using a vacuum rotary evaporator at a temperature maximum of 40 °C. 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 × 10 ml of *n*-hexane – EtOAc (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 °C, 1 h) to hydrolyze the SA-glucoside. The liberated SA was extracted with 3 × 15 ml of *n*-hexane - EtOAc (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.

4.8. 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 3 μ C18 (2) 150 × 4.60 mm equipped with a Security Guard 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.

4.9. Purification using ion exchange chromatography

Purification of SA by ion exchange chromatography was performed based on the system described by Mustafa et al. (2008). 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 4×10 ml of *n*-hexane - EtOAc (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.

4.10. Purification using gel exclusion chromatography

About 30 g 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.

4.11. NMR analysis

^1H 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 ^1H 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, 36,864 scans were recorded with the following parameters: pulse width (30°, 13.0 μs), DS = 4, DW = 13.9 μs, AQ = 1 s and relaxation delay (Dl) = 2.0 s. FIDs were Fourier transformed with LB = 2.20 Hz and SI = 128 kHz.

4.12. Mass spectrometry analysis

Mass spectra were measured on a LTQ–FT Hybrid mass spectrometer (Thermo Fisher Waltham, MA). Spectra were measured in ESI mode, with a source temperature of 275 °C, source voltage of 4 kV and a sheatgas of 35. Chromatograms were recorded in LTQ iontrap SIM mode with a scan range of 127.2 to 147.2 *m/z* for SA and a scan range of 143.1 to 163.1 *m/z* for 2,3-DHBA. Salicylic acid and 2,3-DHBA were dissolved in 90% MeOH and 10% H_2O and directly infused with 10 μl/min. Accurate masses were determined under the same conditions but in FT SIM mode.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2009.01.009.

References

Berg, J.M., Tymoczko, J.L., Stryer, L., Clarke, N.D., 2002. Biochemistry. W.H. Freeman and Company, New York.

Budi Muljono, R.A., 2001. The Isochorismate Pathway as A Route to 2,3-Dihydroxybenzoic Acid in *Catharanthus roseus* Cell Cultures. Leiden University's Thesis, Leiden, The Netherlands, Optima Grafische Communicatie, pp. 69–80.

Budi Muljono, R.A., Scheffer, J.J.C., Verpoorte, R., 2002. Isochorismate is an intermediate in 2,3-dihydroxybenzoic acid biosynthesis in *Catharanthus roseus* cell cultures. Plant Physiol. Biochem. 40, 231–234.

Chong, J., Pierrel, M.A., Atanassova, R., Werck-Reichhart, D., Fritig, B., Saindrenan, P., 2001. Free and conjugated benzoic acid in tobacco plants and cell cultures. Induced accumulation upon elicitation of defense responses and role as salicylic acid precursors. Plant. Physiol. 125, 318–328.

Garcion, C., Métraux, J.P., 2006. Salicylic acid. In: Hedden, P., Thomas, S.G. (Eds.), Plant Hormone Signaling. Blackwell, UK, Oxford, pp. 229–255.

Hatayama, T., Takeno, K., 2003. The metabolic pathway of salicylic acid rather than of chlorogenic acid is involved in the stress-induced flowering of *Pharbitis nil*. J. Plant Physiol. 160, 461–467.

Moreno, P.R.H., van der Heijden, R., Verpoorte, R., 1994. Elicitor-mediated induction of isochorismate synthase and accumulation of 2,3-dihydroxybenzoic acid in *Catharanthus roseus* cell suspension and shoot cultures. Plant Cell Rep. 14, 188–191.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant. 15, 473–497.

Mustafa, N.R., 2007. Retrobiosynthetic Study of Salicylic Acid in *Catharanthus roseus* Cell Suspension Cultures. Leiden University's Thesis, PrintPartners Ipskamp B.V., Amsterdam, The Netherlands, pp. 35–45 and pp. 87–107.

Mustafa, N.R., Kim, H.K., Choi, Y.H., Verpoorte, R., 2008. Single step purification of salicylic acid from *Catharanthus roseus* cell culture (plant material) by anion exchange for NMR analysis. J. Liq. Chromatogr. Relat. Technol. 31, 702–713.

Mustafa, N.R., Verpoorte, R., 2005. Chorismate derived C6C1 compounds in plants. Planta 222, 1–5.

Mustafa, N.R., Verpoorte, R., 2007. Phenolic compounds in *Catharanthus roseus*. Phytochem. Rev. 6, 243–258.

Rajagopalan, I., Jaffe, E.K., 1993. Microbial production and NMR characterization of [2,6,9-^{13}C]chorismate and [1,3,5,8-^{13}C] chorismate. Bioorg. Med. Chem. Lett. 3, 1453–1456.

Scott, K.N., 1972. Carbon-13 nuclear magnetic resonance of biologically important aromatic acids: I. Chemical shifts of benzoic acid and derivatives. J. Am. Chem. Soc. 94, 8564–8568.

Van der Heijden, R., Jacobs, D.I., Snoeijer, W., Hallard, D., Verpoorte, R., 2004. The *Catharanthus* alkaloids: pharmacognosy and biotechnology. Curr. Med. Chem. 11 (22), 607–628.

Verberne, M.C., Brouwer, N., Delbianco, F., Linthorst, H.J.M., Bol, J.F., Verpoorte, R., 2002. Method for the extraction of the volatile compound salicylic acid from tobacco leaf material. Phytochem. Anal. 13, 45–50.

Verberne, M.C., Budi Muljono, R.A., Verpoorte, R., 1999. Salicylic acid biosynthesis. In: Libbenga, K., Hall, M., Hooykaas, P.J.J. (Eds.), Biochemistry and Molecular Biology of Plant Hormones. Elsevier, London, pp. 295–312.

Verberne, M.C., Verpoorte, R., Bol, J.F., Mercado-Blanco, J., Linthorst, H.J.M., 2000. Overproduction of salicylic acid in plants by bacterial transgenes enhances pathogen resistance. Nat. Biotechnol. 18, 779–783.

Weinberg, E.D., 1978. Iron and infection. Microbiol. Rev. 42, 45–66.

Werner, I., Bacher, A., Eisenreich, W., 1997. Retrobiosynthetic NMR studies with ^{13}C-labeled glucose. J. Biol. Chem. 272, 25474–25482.

Wildermuth, M.C., Dewdney, J., Wu, G., Ausubel, F.M., 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defense. Nature 414, 562–565.