Chapter 3

Phenolic compounds in *Catharanthus roseus*

N. R. Mustafa and R. Verpoorte

Division of Pharmacognosy, Section Metabolomics, Institute of Biology

Leiden University, Leiden, The Netherlands

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).
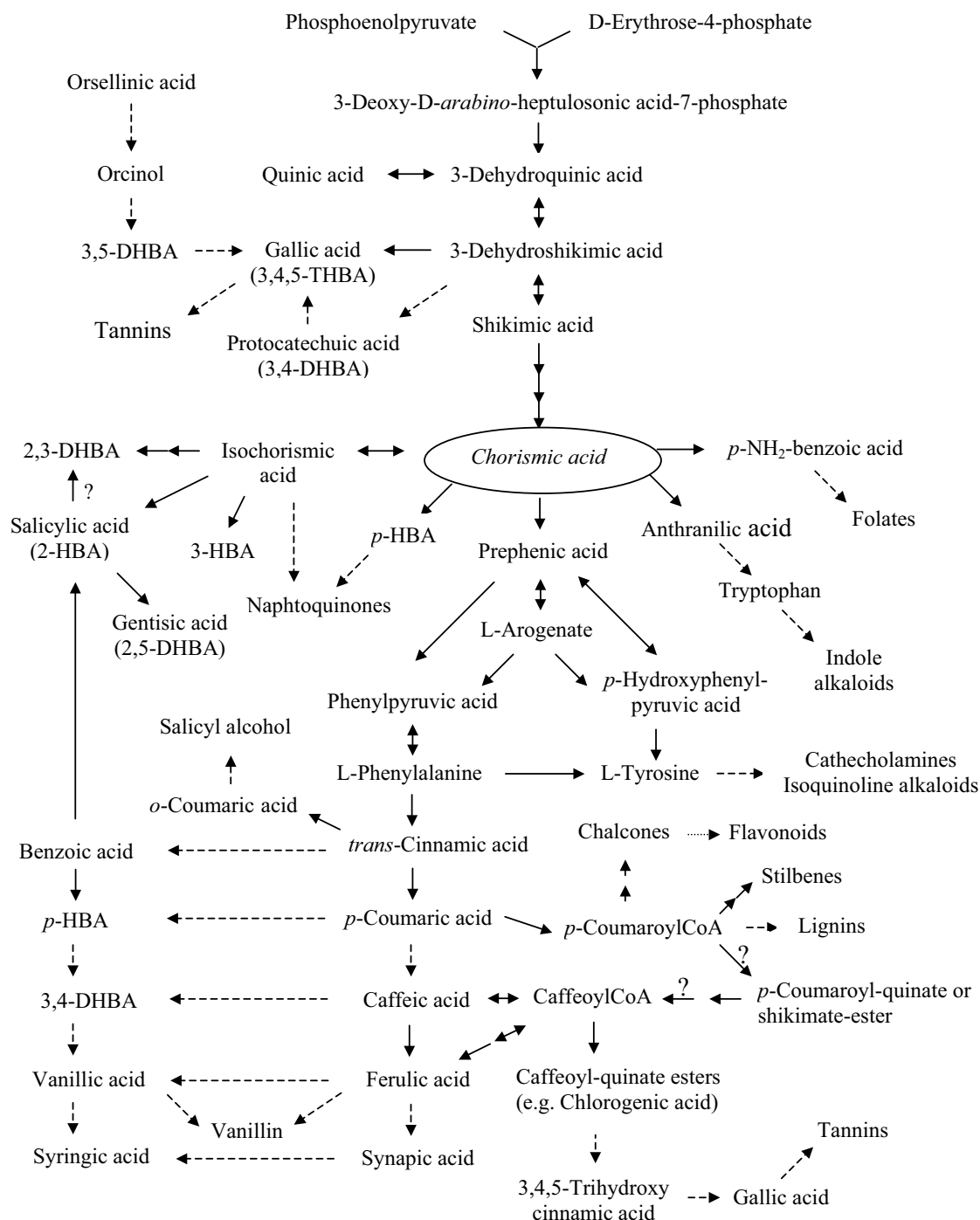

Figure 3.1. The biosynthetic pathway of some phenolic compounds. A small-dashed line means multi-steps reactions.

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).
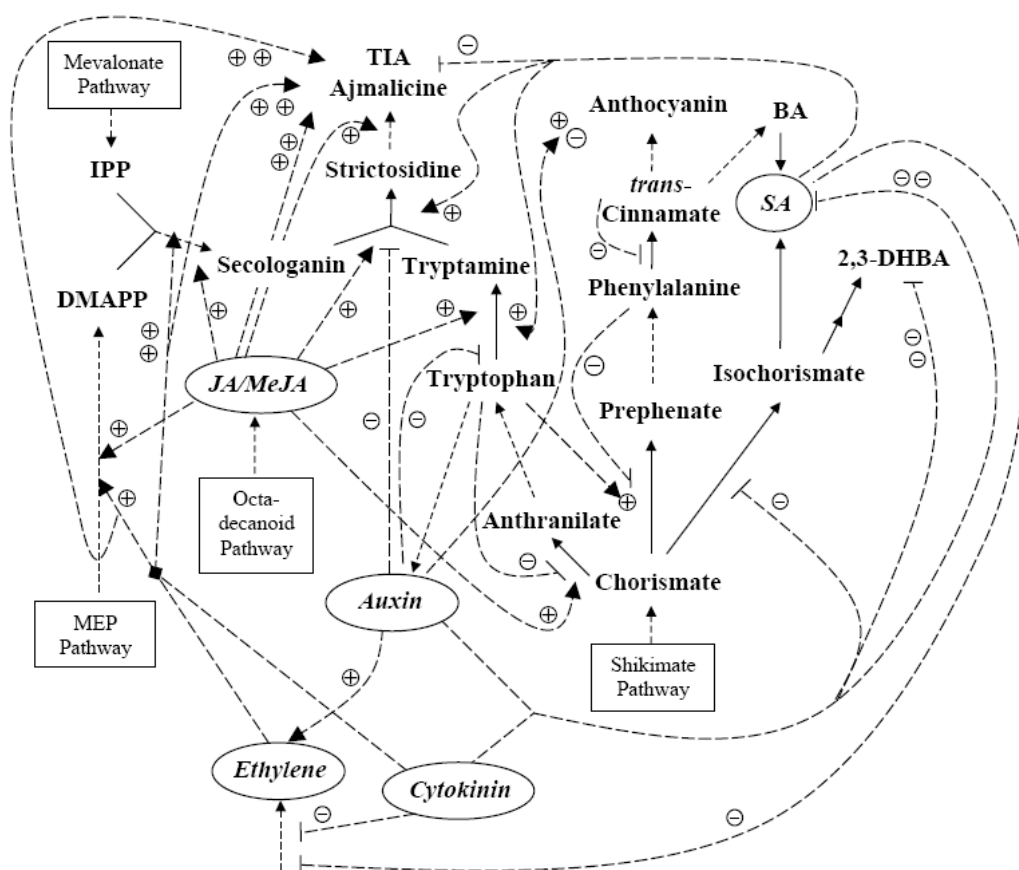

Figure 3.2. Summary of effects reported for various plant hormones and signal compounds in *Catharanthus roseus* cell cultures. A continued line means one-step reaction. A small-dashed line means multi-step reactions. A big-dashed line with + or - indicates activation or inhibition of gene(s) expression, 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.