Metabolomic characteristics of Catharanthus roseus plants in time and space

Qifang, P.

Chapter 3

A simple and rapid HPLC-DAD method for simultaneously monitoring the accumulation of alkaloids and precursors in different parts and different developmental stages of *Catharanthus roseus* plants

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Abstract

A rapid and simple reversed phase liquid chromatographic system has been developed for simultaneous analysis of terpenoid indole alkaloids (TIAs) and their precursors. This method allowed separation of 11 compounds consisting of eight TIAs (ajmalicine, serpentine, catharanthine, vindoline, vindolinine, vincristine, vinblastine, and anhydrovinblastine) and three related precursors i.e. tryptophan, tryptamine and loganin. The system has been applied for screening the TIAs and precursors in *Catharanthus roseus* plant extracts. In this study, different organs i.e. flowers, leaves, stems, and roots of *C. roseus* were investigated. The results indicate that TIAs and precursor accumulation varies qualitatively and quantitatively in different organs of *C. roseus*. The precursors showed much lower levels than TIAs in all organs. Leaves and flowers accumulate higher levels of vindoline, catharanthine and anhydrovinblastine while roots have higher levels of ajmalicine, vindolinine and serpentine. Moreover, the alkaloid profiles of leaves harvested at different ages and different growth stages were studied. The results showed that the levels of monoindole alkaloids decreased while bisindole alkaloids increased with leaf aging and upon plant growth. The HPLC method has been successfully applied to detect TIAs and precursors in different types of *C. roseus* samples to facilitate further study of the TIA pathway and its regulation in *C. roseus* plants.

Introduction

Catharanthus roseus is famous for its diversity of more than 130 terpenoid indole alkaloids (TIAs). Among them the bisindole alkaloids anhydrovinblastine, vinblastine and vincristine, of which the latter two are prescribed for the treatment of various types of cancer (van der Heijden *et al.* 2004), whereas the monoindoles ajmalicine and serpentine are used for their sedative activity in the treatment of hypertensia, as well as their antidiabetic activity (Benjamin *et al.*, 1994; Ahmed *et al.*, 2010; Datta *et al.*, 2010).

With the elucidation of intermediates, enzymes, genes and transcription factors in the TIA pathway, great effort has been made on the metabolic engineering of TIA biosynthesis in *C. roseus* cells and hairy roots for increasing valuable TIAs production by genetic modification (Verpoorte *et al.* 2000; Verpoorte and Memelink 2002; Hughes *et al.* 2004; Zhao and Verpoorte, 2007; Zarate and Verpoorte, 2007). It is crucial to identify and quantify the metabolites and fluxes in the TIA pathways and its related precursor branches. Various analytical techniques and methods have been significantly improved to highly efficient, fast, and accurate high-throughput tools for screening and analyzing qualitatively and quantitatively the profiles of alkaloids in *C. roseus* plants, cell cultures and hairy roots. Thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) are general separation techniques coupled with different types of detectors, such as fluorescence, UV-detection (photo diode array), ESI-MS, MS-MS, NMR and CD, which are useful in identifying and/or quantifying different types of TIAs and precursors (Monforte-González *et al.*, 1992; Choi *et al.*, 2004; Zhou *et al.*, 2005; Ferreres *et al.*, 2010; He *et al.*, 2011). HPLC-UV is a favored technique that can separate, identify and quantify alkaloids due to the efficiency, reproducibility, availability in most laboratories, simple sample preparation, and ability of both qualitative and quantitative analysis. However, the capacity of TIAs separated in one run by one HPLC method is always limited from two to seven alkaloids (Hisiger and Jolicoeur, 2007). Two different HPLC methods were developed to separate and analyse six TIAs and four precursors respectively (Tikhomiroff and Jolicoeur 2002). However, it would be better to simultaneously measure both TIAs and

precursors in the complex matrix of *C. roseus* plant extracts by one simple method.

Generally, TIAs and precursors are analyzed by two different HPLC separation methods due to their different characteristics. The most efficient method for precursor analysis was reported to be a separation of five compounds from an extract (Dagnino *et al.*, 1995 and 1996). The published methods for TIA analysis were able to separate two to seven alkaloids (Hisiger and Jolicoeur, 2007). However, there have been no studies reporting a single method to separate both precursors and TIAs in a sample extract. It is thus necessary to achieve further improvements to obtain a simple and fast HPLC technique for analysis of *C. roseus* alkaloid metabolism. Here, we were inspired by the work of Tikhomiroff and Jolicoeur (2002) to develop such a method.

Terpenoid indole alkaloid biosynthesis has a tight relationship with tissue differentiation and cell compartmentation, which limits the use of *C. roseus* tissue cultures (cells and hairy roots) in the industrial production of valuable alkaloids. Bisindoles, such as vincristine and vinblastine, are synthesized in aerial parts of the plant, mainly in leaves (De Luca and Laflamme, 2001), whereas vingramine and methylvingramine have been reported to be detectable only in seeds of *C. roseus* (Jossang *et al.* 1998). Ajmalicine, serpentine, hörhammericine, lochnericine, 19-hydroxytabersonine, 19-*O*-acetyl-hörhammericine and echitovenine are mostly present in roots (Shanks *et al.* 1998; Laflamme *et al.* 2001; Rodriguez *et al.* 2003), and are rarely detected in cell cultures (Kutney *et al.* 1980). Vindoline, one of the two precursors of the bisindole alkaloids, is produced in the green parts of the plant and does not exist in the roots, hairy roots or cell cultures (He *et al.* 2011). Moreover, the activity of key enzymes LAMT and 16-hydroxytabersonine O-methyltransferase (16OMT) showed a negative relation with leaves age (Murata *et al.*, 2008). Previous publications reported that the alkaloid content changed coinciding with the different developmental stages of *C. pusillus* plants (Zarate *et al.*, 2001). However, *C. pusillus* does not produce the bisindoles vinblastine and vincristine.

In this study, a single HPLC-UV method was developed to simultaneously analyze eight TIAs (vindoline, catharanthine, ajmalicine, serpentine, vindolinine, anhydrovinblastine, vinblastine and vincristine) and three precursors

(tryptophan, tryptamine and loganin) in *C. roseus* plant materials. The contents of TIAs and precursors were analyzed and compared among flowers, young leaves, old leaves, stems and roots of *C. roseus*. In addition, the metabolic profile of TIAs and precursors in leaves of different ages and growth stages were studied in order to evaluate the accumulation and distribution of TIAs in space and through time.

Materials and methods

Chemicals

The HPLC grade acetonitrile and methanol was bought from Carl Roth GmbH (Karlsruhe, Germany) and Biosolve B.V. (Valkenswaard, The Netherlands), respectively. Methanol and ethanol were of analytical reagent (AR) grade from Biosolve B.V. Loganin and vindoline were bought from PhytoLab, Vestenbergsgreuth, Germany. Tryptamine was purchased from Aldrich Chemical, Milwaukee, WI, USA. Tryptophan, vindolinine and ajmalicine were purchased from Sigma-Aldrich, St. Louis, MO, USA. Serpentine was purchased from Carl Roth GmbH. Catharanthine, anhydrovinblastine, vinblastine and vincristine were from Pierre Fabre, Gaillac, France. $NaClO_2$ was purchased from Sigma-Aldrich.

HPLC conditions

The chromatographic system was an Agilent Technologies 1200 series, consisting of a G1322A vacuum degasser，a G1310A pump, a G1329A auto-sampler，and a G1315D diode array detector (Agilent Techologies Inc). The column was an Agilent Eclipse XDB-C18 column (4.6×250 mm, 5 μm particle size) (Agilent Techologies Inc., Santa Clara, CA, USA), coupled with a SecurityGuard^{TM} column (Phenomenex, Torrance, CA, USA).

The mobile phase consisted of a mixture of 5 mM Na_2HPO_4 (pH adjusted to 6 with HCl) (solvent A) and methanol (solvent B) at a flow rate of 1.5 ml per min. The eluent profile (solvent A / solvent B) was set to a linear gradient from 86:14 to 14:86 at 0-26 min, an isocratic mode (14:86 v/v at 26-30 min), a linear gradient from 14:86 to 86:14 at 30-35 min, an isocratic elution with 14:86 (v/v)

at 35-37 min. The injection volume was 30 µl.

Identification and quantitation

Standard solutions were prepared following Tikhomiroff and Jolicoeur (2002). The quantification was performed using six levels of external standards. Level 6 consisted of a dilution (1:50, v/v) of stock solution (5 mg/mL) in methanol. Levels 5 to 1 were obtained by dilution of level 6 by a factor 2, 4, 8, 16 and 32 respectively. The ranges obtained were 2-4 µg/ml to 64-128 µg/ml depending on the concentration of each compounds' stock solution. Identification of alkaloids from the plant extracts was performed by comparison of the UV spectra and retention time with those of authentic standards. Each calibration curve was obtained by making each concentration in triplicates and measuring them. Test samples were analyzed in triplicate for quantification using the calibration curves of the standards. The limit of detection and extraction yield was measured as described by Tikhomiroff and Jolicoeur (2002).

Plant materials

Seeds of *C. roseus* (cv. Pacific Cherry Red) were purchased from PanAmerican Seed Company (West Chicago, IL, U.S.A.). The seeds were surface sterilized in 75% (v/v) ethanol for 2 min and 5% (v/v) $NaClO_2$ for another 5 min. Subsequently, seeds were washed five times with sterile distilled water and germinated on Petri dishes containing MS (Murashige and Skoog, 1962) basal medium. Cultures were grown under 16 h light and 8 h dark photoperiod at 25 ± 2 ℃. After germination for 2 weeks, seedlings were transplanted into soil and cultivated in the greenhouse. When blooming, flowers, leaves, stems and roots of the plants were separately harvested and directly frozen in liquid nitrogen for the following experiments. Some plants of another batch were collected at 4:00 pm of the 30th, 44th, 62th, 79th and 99th days after germination in order to observe the change of alkaloid content during the growth stages. Samples of each observation group were meassured in triplicate.

Sample preparation

Freshly collected sample materials were ground in liquid nitrogen using mortar and pestle. Subsequently, the samples were lyophilized for 72 hours. The dried sample (30 mg) was put in a micro-tube and extracted with 1 ml methanol by vortexing and a sonication (30 min) in an Ultrasonic bath (DL-60D). Then, samples were centrifuged at 12,000 rpm for 10 min at room temperature and the supernatant was filtered with 0.45 μm needle type PTFE membrane filter prior to HPLC analysis.

Data statistical analysis

All experiments were conducted with three replicates. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range (DMRT) test. The values are mean ± standard deviation for three samples in each group. Level of significance set at 0.05 (Alpha<0.05) was considered as significant. The ANOVA for all the data was performed by SPSS (version 20.0, Chicago, IL, USA).

Results and discussion

Separation of standards

The HPLC method was developed for simultaneous analysis of both TIAs and their precursors. This method is based on the work of Tikhomiroff and Jolicoeur (2002). Methanol and acetonitrile (ACN) are common organic solvents used as mobile phase in reversed phase-HPLC. Considering the global shortage of ACN, methanol was used to develop a single HPLC method for separation of the TIAs and precursors in this study. Accuracy of the pH was critical to achieve the separation (Tikhomiroff and Jolicoeur, 2002). The composition of mobile phase was optimized for proportions of the 5 mM of potassium phosphate buffer pH 6 (solvent A) and methanol (solvent B), which provided a good separation of eight TIAs (serpentine, vindoline, vincristine, catharanthine, vinblastine, ajmalicine and anhydrovinblastine) and three precursors i.e. tryptophan, tryptamine and loganin. Figure 1 shows the

chromatogram of the mixture of the reference compounds. Retention time, UV wavelength, and quantification range of standard compounds are presented in table 1.

Table 1. Detection parameters of indole alkaloids and precursors.

Metabolite	**Retention time (min)**	**Wavelength (nm)**	**Test range (µg/ml)**	**Extraction yield (%)**
Tryptophan	5.211	280	6.07 - 109.99	66.09 ±5.6
Tryptamine	7.654	280	1.43 - 94.84	109.22 ±3.2
Loganin	10.332	238	4.28 - 102.12	85.87 ±4.1
Serpentine	17.553	306	2.95 - 83.17	23.96 ±3.6
Vindolinine	19.752	220	3.04 - 85.39	64.95 ±4.7
Vindoline	24.002	220	3.20 - 99.94	58.61 ±5.2
Vincristine	24.934	220	3.62 - 95.68	72.64 ±7.2
Catharanthine	25.445	280	1.46 - 99.60	36.38 ±4.8
Vinblastine	26.071	220	0.28 - 33.51	64.95 ±5.1
Ajmalicine	26.362	280	0.17 - 56.10	106.81 ±10.3
Anhydrovinblastine	29.784	220	3.77 - 98.38	65.53 ±3.8

Results are the mean of 3 replicates ± standard deviation.

Under the described HPLC conditions, a linear relationship between the concentration of precursors and TIAs and their UV absorbance at 254 nm was obtained. The correlation coefficient for the standard curves (r^2) exceeded 0.99 (Table 2). Based on the standard deviation (SD) of the response and the slope (S) of the calibration curves, the limit of detection (LOD) was calculated according to the formula (Shabir, 2003): LOD = 3.3 (SD/S) and the limit of quantification (LOQ) was calculated following the formula: LOQ = 10(SD/S). The SD of the response was determined based on the y-intercepts of regression lines. Results are shown in Table2.

Table 2. Equations for regression lines, correlation coefficients, limit of detection (LOD) and limit of quantification (LOQ) of the HPLC method for alkaloids.

Compounds	**Equation**	**r^2**	**LOD (µg/ml)**	**LOQ (µg/ml)**
Tryptophan	y = 357409x - 59.649	0.9953	0.55	1.67
Tryptamine	y = 322821x + 41.738	0.9943	0.43	1.29
Loganin	y = 203553x - 24.251	0.9987	0.39	1.19
Serpentine	y = 3000000x - 531.15	0.9993	0.58	1.77
Vindolinine	y = 98946x - 11.484	0.9934	0.38	1.16
Vindoline	y = 704511x + 25.892	0.9997	0.12	0.37
Vincristine	y = 157734x - 8.3234	0.9989	0.17	0.53
Catharanthine	y = 257481x + 104.35	0.9998	1.34	4.05
Vinblastine	y = 418103x + 91.45	0.9992	0.72	2.19
Ajmalicine	y = 1000000x - 1283.9	0.992	4.24	12.84
Anhydrovinblastine	y = 382040x + 40.505	0.9982	0.35	1.06

Repeatability was checked by analyzing five times the same sample, by the same analyst, within the same day. Relative standard deviation (RSD) varying between 1.19% and 2.95% indicated that the repeatability of the procedure was good. Intermediate precision determined by different analysts on three separate days was also found satisfactory (RSD ranging from 1.58% to 3.10%).

The extraction yield was measured to validate the extraction method for each studied compound and shown to be dependent on the compound (Table 1). The recoveries of most compounds (except serpentine) were higher than those reported by Tikhomiroff and Jolicoeur (2002), indicating that the method was appropriate for screening of both TIA and precursors in *C. roseus* plants.

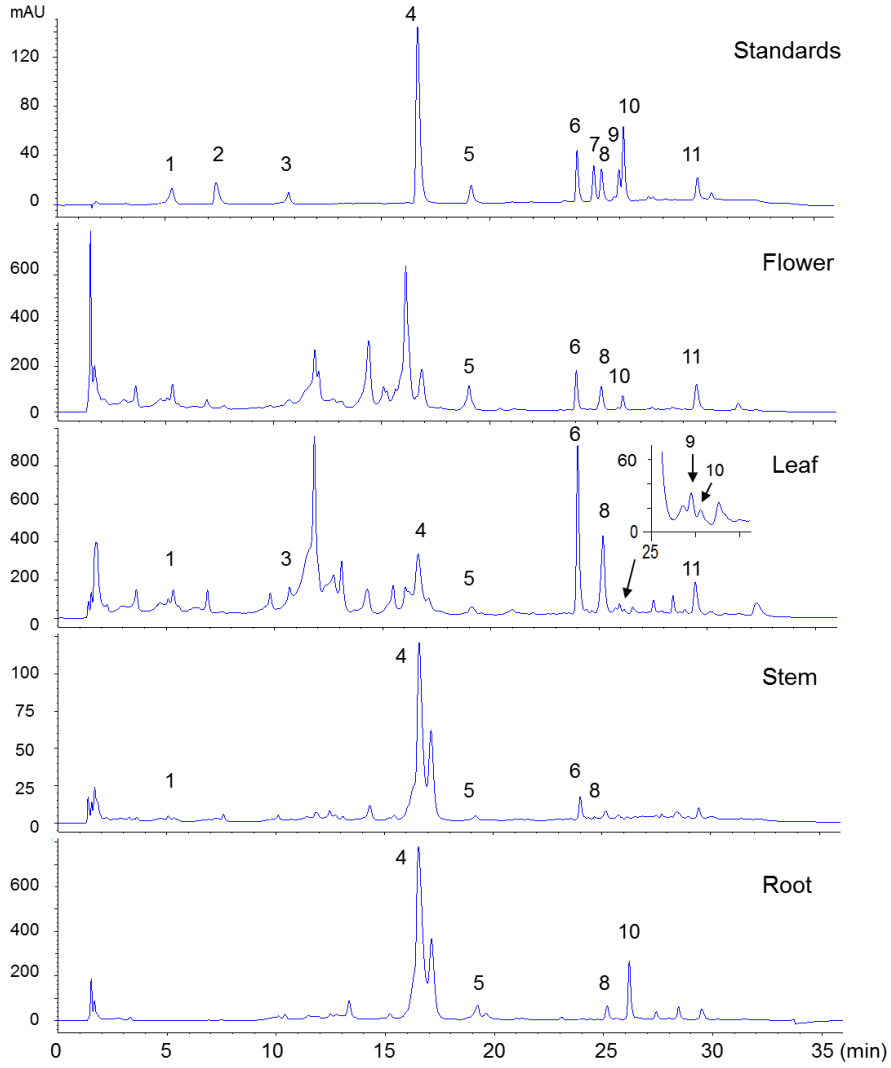

Fig. 1 HPLC-DAD chromatograms of the standard mixtures (TIAs and precursors) and methanol crude extracts of flower, leaf, stem and root of *Catharanthus roseus* at wavelength of 254 nm. 1. Tryptophan; 2. Tryptamine; 3. Loganin; 4. Serpentine; 5. Vindolinine; 6. Vindoline; 7. Vincristine; 8. Catharanthine; 9. Vinblastine; 10. Ajmalicine; 11. Anhydrovinblastine.

The profile of alkaloids and precursors in different organs

The extraction and the chromatography method were evaluated by extraction of different organs i.e. flower, leaf, stem, and root of *C. roseus* with methanol and analyzing the extracts using the HPLC system. Figure 1 shows the chromatograms of crude extracts of *C. roseus* different organs. Most of the alkaloids were detected in the leaves, where also the precursors tryptophan and of the iridoid pathway are found. The leaf TIAs included ajmalicine, serpentine, vindoline, catharanthine, as well as the bisindole alkaloids of anhydrovinblastine and vinblastine. Vindolinine, vindoline, catharanthine, ajmalicine, and anhydrovinblastine were detected in the flowers. In contrast, no bisindole alkaloids were detected in *C. roseus* stems, but tryptophan, serpentine, vindoline and catharanthine were identified in this organ. Similarly, no bisindole alkaloids were found in the roots, but serpentine, vindolinine, catharanthine and ajmalicine were clearly detected. Furthermore, quantitative analysis was performed to evaluate the levels of precursors and TIAs in different organs (Table 3).

Table 3. The levels of TIAs and precursors in flower, leaf, stem and root of *Catharanthus roseus* during flowering. *-: not detected

Compound (mg/g DW)	**Flower**	**Leaf**	**Stem**	**Root**
Tryptophan	-	[a] 0.23 ±0.01	[a] 0.03 ±0.01	-
Tryptamine	-	-	-	-
Loganin	-	1.14 ±0.08	-	-
Serpentine	-	[a] 0.40 ±0.09	[a] 0.16 ±0.02	[a] 0.73 ±0.25
Vindolinine	[a] 0.67 ±0.32	[a] 0.51 ±0.23	[a] 0.23 ±0.04	[b] 3.38 ±0.61
Vindoline	[a] 0.22 ±0.09	[b] 1.29 ±0.36	[a] 0.06 ±0.01	-
Vincristine	-	-	-	-
Catharanthine	[a] 0.16 ±0.08	[c] 1.54 ±0.49	[a] 0.03 ±0.01	[b] 0.81 ±0.25
Vinblastine	-	0.05 ±0.02	-	-
Ajmalicine	[a] 0.09 ±0.02	[a] 0.04 ±0.02	-	[b] 0.30 ±0.06
Anhydrovinblastine	[a] 0.49 ±0.10	[a] 1.30 ±0.51	-	-

[a, b, c]: significant difference (Alpha<0.05 by ANOVA)
DW: dry weight; Results are the mean of 3 replicates ± standard deviation.

The results showed that leaves contain the highest levels of tryptophan, vindoline, catharanthine, and anhydrovinblastine, among which the accumulation of vindoline and catharanthine were significantly higher in leaves than in other organs. While the accumulation of vindolinine, ajmalicine and serpentine were found highest in the roots. Both flowers and stems contained a relatively low level of TIAs and precursors. For the different organs that have been analyzed, vinblastine was only detected in the leaves, while tryptamine and vincristine were not detected in any organ. Transcriptome analysis showed a significant difference of gene expression pattern in *C. roseus* leaves and roots (Shukla *et al.*, 2006). It was reported that complex alkaloid fluctuations were recorded from organ to organ in *C. pusillus*, which also belongs to the genus *Catharanthus* (Zarate *et al.* 2001).

Alkaloids in leaves of different age

Leaves at different positions along the stem are of different age. Younger leaves are in the upper-3-layers from the top and the older ones are in the lower-3-layers from the bottom (El-Sayed and Verpoorte 2005). The leaves in the middle layers (excluded from upper- and lower- layer leaves) are at an age in between. In this study, the levels of TIAs and precursors were measured at the three different layers of leaves along the stem of *C. roseus* plants (Fig. 2), which represents different leaf ages. The levels of TIAs (serpentine, vindolinine, vindoline, catharanthine and ajmalicine) decreased while the precursors loganin and tryptophan increased as *C. roseus* leaves were aging. Bisindole alkaloids anhydrovinblastine and vinblastine showed the highest level in the middle-layer leaves with a fluctuating pattern of accumulation. Statistical analysis showed that the levels of monoindole alkaloids were significantly higher in upper-layer leaves, and precursor levels were significantly higher in lower-layer leaves, while the levels of bisindole alkaloids were significantly higher in middle-layer leaves. The difference of accumulation pattern between monoindole and bisindole alkaloids indicates that their levels were not directly correlated to each other. Previous studies reported that the biosynthesis of vinblastine is related with the age of leaves (Naaranlahti *et al.* 1991). Moreover, it was revealed that the activity of biosynthetic enzymes (NMT, 16-OMT and LAMT) decreased as the leaf age of *C. roseus* increased (Murata *et al.*, 2008). Our results indicate

that when leaves are getting older, the alkaloid precursors are accumulating but seem not to be available for the biosynthesis of monoindole alkaloids as they show a reduction of their level. Bisindole alkaloids accumulated in aging leaves. The production of defense compounds is highest in younger leaves as these leaves have the highest potential for photosynthesis for the plant (Smith, 1966; Mc Key, 1974; van Dam *et al.*, 1995; Barto and Cipollini, 2005). This results thus in the highest levels in the youngest leaves. But the total absolute amount of alkaloid in a leave is more or less the same for young and old leaves. The experiments done support this scenario for alkaloid levels.

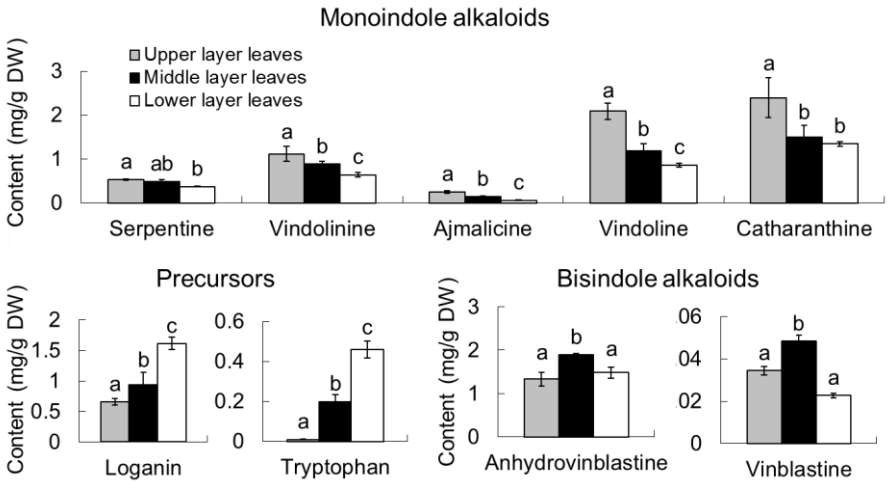

Fig. 2 Terpenoid indole alkaloids and precursors content in *Catharanthus roseus* leaves at different ages. Layer from upper to lower represents leaves getting old with aging. Different letters indicate significant difference (Alpha<0.05 by ANOVA). Results are the mean of 3 replicates ± standard deviation. DW: dry weight.

Alkaloids in the growth stage of C. roseus plants

The *C. roseus* plants got mature and began to flower at around 65 ~ 75 days after sowing. The flowering stage lasted for at least 3 months. In this study, we observed the TIA levels in the leaves from the seedling stage to flowering stage (Fig. 3).

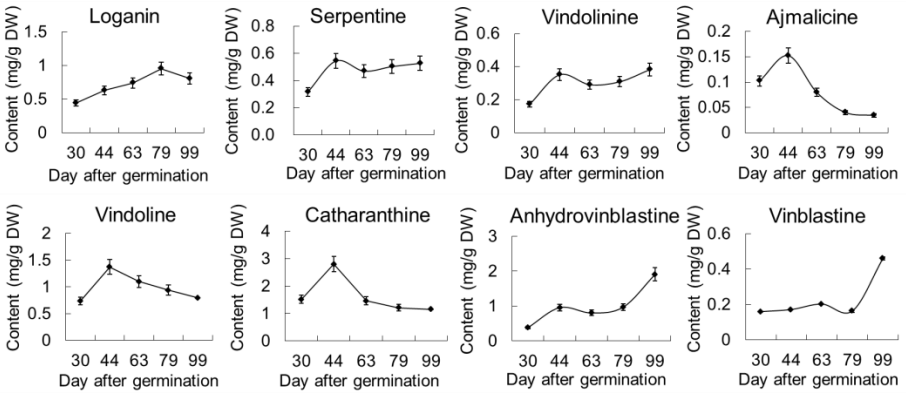

Fig. 3 Levels of TIAs and loganin in *Catharanthus roseus* leaves during growth stage. Results are the mean of 3 replicates ± standard deviation. DW: dry weight.

The major two TIAs in *C. roseus* leaves were vindoline and catharanthine, which are the precursors of bisindole alkaloids. Accumulation of vindoline and catharanthine at the seedling stage, tended to increase and reached the highest levels at day 44 (2.74 μg/g DW and 5.96 μg/g DW) before flowering, and subsequently declined with the flowering time. Ajmalicine levels had a similar pattern with that of vindoline and catharanthine, reaching the highest level at day 44 (0.15μg/g DW). In *C. pusillus*, samples of the cotyledons had the highest value of ajmalicine (Zarate *et al.*, 2001). Other TIAs, such as serpentine and vindolinine, showed different patterns. The contents of serpentine and vindolinine increased to the value of 0.54 μg/g DW and 0.35 μg/g DW respectively at the seedling stage and remained similar to the flowering stage. The accumulation patterns of the bisindole alkaloids anhydrovinblastine and vinblastine were totally different from their precursors. Both of them accumulated at the flowering time and displayed the highest levels (1.90 μg/g DW and 0.46 μg/g DW) at day 99 at the flowering stage. The alkaloid yields depend on the developmental stage of plants (Verpoorte, 1998; Zarate *et al.*, 2001). These results indicate that before flowering the *C. roseus* plants already actively accumulate various important TIAs possibly as defence for the growing plant (Luijendijk *et al.*, 1996; Meisner *et al.*, 1981; van Dam *et al.*, 1995). Since bisindole alkaloids increased as leaves aged (El-Sayed and Verpoorte, 2005),

the mature- and flowering plants produced more bisindole alkaloids than the seedlings.

Conclusion

An HPLC method was developed and validated for simultaneously determination of eight TIAs and three precursors in *C. roseus*. The levels of TIAs and their precursors varied between tissues and upon growth of *C. roseus* plants. Flowers and leaves produced more types of TIAs than stems and roots did. Leaves were the major site to accumulate vindoline, catharanthine and anhydrovinblastine whilst roots accumulated most vindolinine, ajmalicine and serpentine. Vinblastine was only detected in leaves. With leaves aging the levels of monoindole alkaloids decreased, but the levels of their precursors tryptophan and loganin increased. The middle-age leaves constituted the major repository site of bisindole alkaloids. Upon the blooming of plants, the levels of vindoline, catharanthine and ajmalicine increased before flowering and decreased during flowering, whilst anhydrovinblastine and vinblastine accumulated at the flowering time. Blooming didn't affect the production of serpentine and vindolinine.

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