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Full Length Research Paper

Variation in coumarin accumulation by stem age in *Dendrobium thyrsiflorum* (Orchidaceae) at different developmental stages

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In this study, laser scanning confocal microscopy was applied to determine the localization and relative quantity of coumarins in stems of *Dendrobium thyrsiflorum* Rchb. f. (Orchidaceae) when plants entered profuse flowering and initial fruit period during reproductive growth stage. Stems at the two growth stages were collected respectively. Distribution of coumarins in the top, middle and basal parts of each stem sample of 1, 2 and 3-year-old were observed by laser scanning confocal microscope. ANOVA and Tukey's test were employed in the statistical analyses. The results showed that coumarins was located mainly in vascular bundle and its outer fiber cell wall, ground tissue cell wall and nearby, wall of epidermal cell and hypodermis cell as well. Statistical analyses indicated the significant or great significant difference presented in every part of stem of different ages in different growth periods except in the middle part during profuse flowering time. The content of coumarins reached its highest level when flowers profuse whereas at initial fruit stage that of coumarins was the lowest. Harvest activities of *D. thyrsiflorum* should be carried out when plants entered profuse flowering period in order to obtain abundant courmarins.

Key words: *Dendrobium thyrsiflorum* Rchb. f., stem, coumarins, variation, developmental stage, LSCM (laser scanning confocal microscopy).

INTRODUCTION

Being relatively plenty in the wild and easy bred, *Dendrobium thyrsiflorum* Rchb. f. (Orchidaceae) is one of the main originals of Shi-hu being a famous traditional Chinese medicine (TCM) and used as remedies for nourishing yin and removing heat-evil (Zhang et al., 2005; Pharmacopoeia Commission of the People's Republic of China, 2005edn). Wrigley's (1960) extract from the plant leaf contained coumarins. This active component has multiple biological functions, such as anti-HIV, anti-tumor, anti-hypertension, anti-arrhythmia, anti-osteoporosis, assuaging pain, preventing asthma and antisepsis; it is even used as a model fluorescent dye. Although many investigations have dealt with the chemical structure and biological functions (Zhanf et al., 2005; Shenoy et al., 2006), few studies have focused on its localization and variation at different stages in different plant organs.

In our previous papers, we once reported the localization and relative quantity of coumarins in stems and roots of *D. thyrsiflorum* from different age collected in February when it was growing in vegetative period (Zheng et al., 2005a, b). In order to provide a comprehensive basis for evaluating and utilizing the medicinal materials, variation in coumarin accumulation by stem age in the plant at different developmental stages was further studied by using histochemical analysis together with laser scanning confocal microscopy (LSCM).

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MATERIALS AND METHODS

Samples (*D. thyrsiflorum* Rchb. f.) were collected in Simao, Yunnan province, China. All those plants were cultured in the greenhouse at China Pharmaceutical University (Nanjing, China) to provide materials for study. Stems of various ages (1, 2, and 3 year old) were taken separately while plants entered profuse flowering period and initial fruit period. Voucher specimens were identified by the authors and deposited in the herbarium at the College of Traditional Chinese Pharmacy, China Pharmaceutical University.

The materials were taken from top, middle and base of fresh stems. Small blocks (about 5 mm^3) were embedded at – 20℃. The blocks were sectioned with Leica CM 1900 cryostat and observed with LSCM. 364 nm excitation lines were chosen as suitable wave length for the selected experiments after being detected by fluorescence spectrophotometer. Images were taken from a Zeiss Axiovert S100 microscope equipped with a Bio-Rad MRC-1024 ES confocal laser-scanning unit. Vascular bundle under 10 - 30 view was chosen randomly and photographed to record its mean/total pixel intensity. ANOVA and Tukey’s test were employed in the statistical analyses. Histograms of different developmental stage were given accordingly.

RESULTS

Distribution of coumarins

Reproductive growth stage 1: profuse flowering period

In 1 year old stem of *D. thyrsiflorum*, coumarins distributed equally in the fundamental tissue cells and cell wall and cavity of the vascular bundles at stem base (Figure 1-1a,b). The same happened in the middle stem except the distribution was unequal (Figure 1-2a,b). There was spread of coumarins in fundamental tissue cells and xylem in vascular bundles in top parts of the stem (Figure 1-3a,b).

At 2 year old stem base, the similar distribution occurred and the phloem cell wall had stronger fluorescence light (Figure 1-4a,b). In the middle, vascular bundle cell wall, outer fiber group, ground tissue cell wall and cavity all had strong fluorescence light (Figure 1-5a,b). At stem top, coumarins existed in inner vascular bundle, outer fiber cell wall, fundamental tissue cell wall and cavity near the epidermis (Figure 1-6a,b).

At the base of 3 year old stem, fluorescence light was strong in vascular bundle cell wall and fundamental tissue cell wall whereas there was none or a few in the cell cavity (Figure 1-7a,b). The middle stem, inner vascular bundle, outer fiber group cell wall and cavity had more coumarins while the component diffused in the fundamental tissue cell (Figure 1-8a,b). At stem top, vascular bundle and its surrounding ground tissue cell wall had coumarins and fluorescence light appeared clearly in outer fiber group and cell corner (Figure 1-9a,b).

Reproductive growth stage 2: Initial fruit period

There was little fluorescence light at 1 year-old stem base (Figure 2-1a,b), a little at the top (Figure 2-3a,b) and concentrated relatively in the middle in vascular bundle and ground tissue cell corner (Figure 2-2a,b). At the top of 2 year-old stem (Figure 2-6a,b), there was a little fluorescence light whereas there was little at the base (Figure 2-4a, b) and in the middle (Figure 2-5a,b). Fluorescence light was wholly faint in 3 year old stem. There was only a small quantity of coumarins distributing in the cell wall, cell corner and cavity around the wall (Figure 2-7a,b to -9a,b). Overall, the fluorescence light was so weak at initial fruit period even transmission effect was used in the figures.

Change of relative content of coumarins

Data from nine parts of the plant materials, including base, middle and top in 1, 2 and 3 year-old stem, were analyzed. Table 1 and Figure 3 showed the results at profuse flowering stage whereas Table 2 and Figure 4 showed the results at initial fruit period.

DISCUSSION

No matter what developmental phases (vegetative or reproductive growth stage), coumarins were present in different stem parts from different age located mainly in vascular bundle and its outer fiber group, ground tissue cell wall or cavity and its nearby, wall of epidermal cell and hypodermis cell as well.

During profuse flowering period, according to ANOVA and Tukey’s test, significant difference was great at stem base and top ($P < 0.01$). But in the middle of stem, significant difference was not great ($P = 0.136$). The mean pixel intensity value of every stem part from different age was as follows (from high to low) (mean pixel intensity ± standard error, unit: pixel intensity): 3 year old stem top (174.61 ± 9.03); 1 year old stem base (168.22 ± 16.63); 2 year old stem base (135.87 ± 29.45); 2 year old stem top (133.85 ± 20.26); 3 year old stem middle (128.50 ± 31.08); 2 year old stem middle (126.00 ± 28.33); 3 year old stem base (110.29 ± 9.03); 1 year old stem middle (107.57 ± 31.12); and 3 year old stem top (87.92 ± 18.41).

At initial fruit period, great significant difference existed in every part of the stem ($P < 0.01$) according to ANOVA and Tukey’s test. The mean pixel intensity value of every stem part from different age was as follows (from high to low) (mean pixel intensity ± standard error, unit: pixel intensity): 1 year old stem top (7.35 ± 0.44); 1 year old stem middle (6.72 ± 1.59); 2 year old stem top (5.78 ± 1.01); 3 year old stem middle (1.94 ± 0.37); 3 year old stem top (0.45 ± 0.05); 3 year old stem base (0.16 ± 0.02); 1 year old stem base (0.06 ± 0.00); 2 year old stem base (0.06 ± 0.02); and 2 year old stem middle (0.01 ± 0.00).

In fact, either mean pixel intensity or total pixel intensity could all reflect the relative content of coumarins in stem of *D. thyrsiflorum*. The two kinds of different pixel intensity showed the same results. In order to make the conclusion more convincingly, total pixel intensity was used as unit of relative content of coumarins and Figures 5 - 7 show the dynamic content change in different stem parts (which is stem base, middle and top) from different age (which is 1,

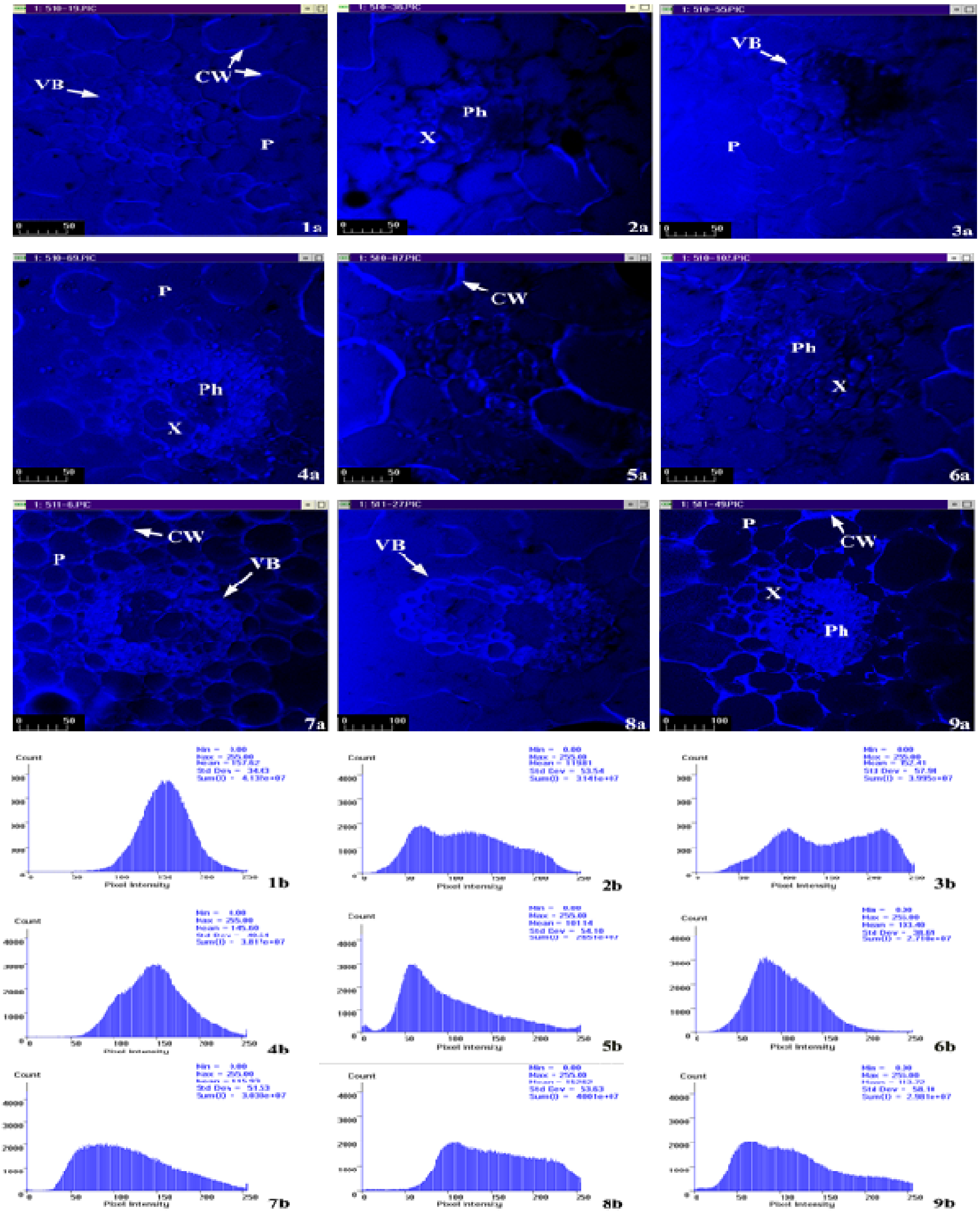

Figure 1. Localozation and relative quantity of coumarins in stem of *Dendrobium thyrsiflorum* Rchb. f when flowers blooming (a: Photo under LSCM; b: Show pixel intensity; CW: cell wall; P: parenchyma; Ph: phloem; VB: vescular bundle; X: xylem).

2 and 3 year old). No matter how old the medicinal plant was, the content of coumarins reached its highest level during profuse flowering period and the lowest during initial fruit period, and the content was intervenient during vegetative period. ANOVA analysis on total fluorescence intensity of *D. thyrsiflorum* from different developmental phases showed the same results (Table 3).

Conclusion

Based on these findings, harvest activities of *D. thyrsiflo-*

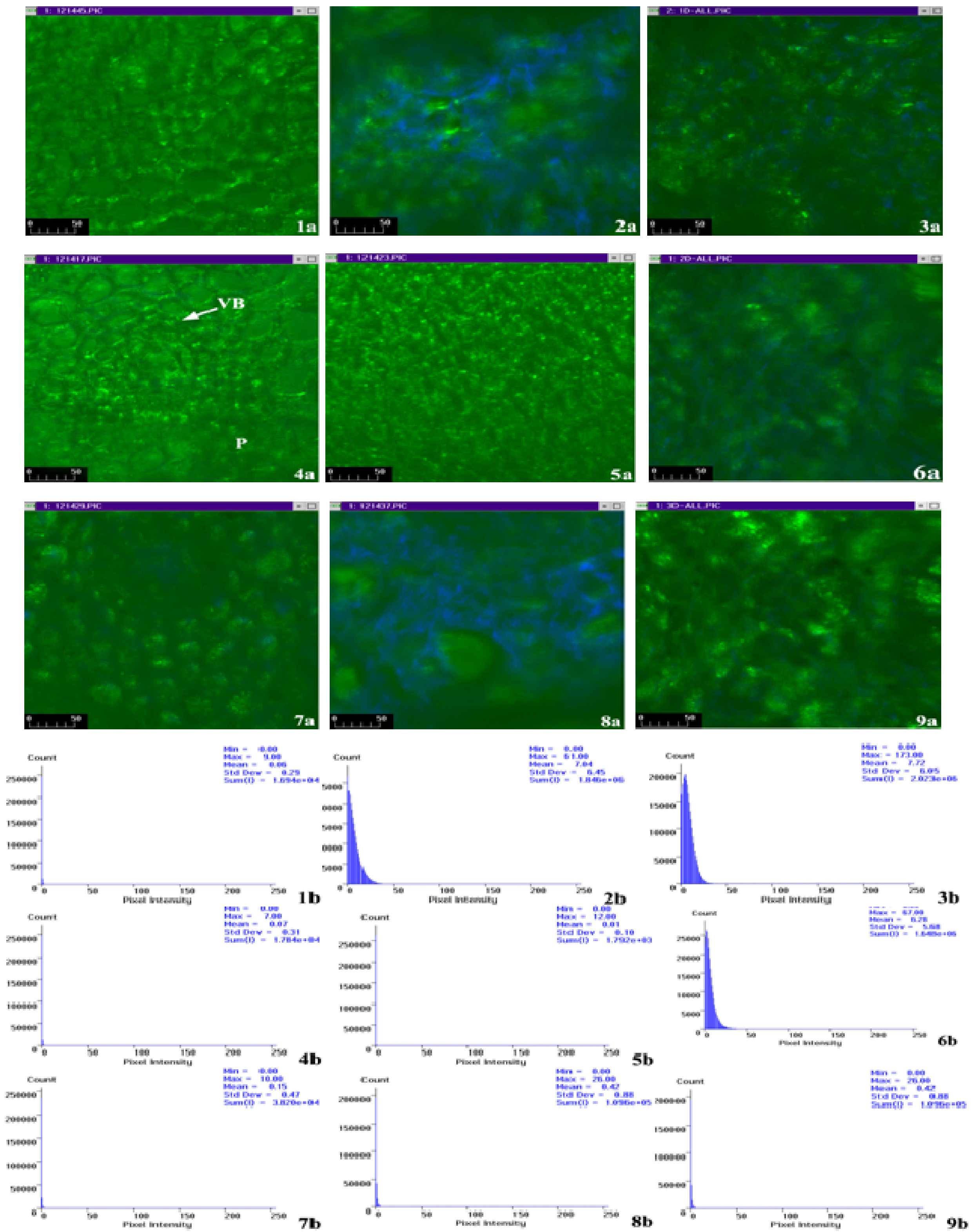

Figure 2. Localization and relative quantity of coumarins in stem of *Dendrobium thyrsiflorum* Rchb. f when flowers are dying (a: Photo under LSCM with transmission effect; b: Show pixel intensity; VB: vescular bundle).

orum should be carried out to obtain abundant courmarin during profuse flowering time. Laser scanning confocal microscopy has been developed rapidly and widely applied in morphological description and ion imaging of

Table 1. ANOVA analysis and Tukey's test on different parts in the stem of *Dendrobium thyrsiflorum* from different age during flowers blooming.

Age	Base of stem	Middle of stem	Top of stem
1-year old stem	168.22[a]	107.57	174.61[a]
2-year old stem	135.87[b]	125.99	133.85[b]
3-year old stem	110.29[c]	128.50	87.92[c]
value of significant difference (*P*)	<0.01	0.136	<0.01

Significant difference tests: a>b>c.

Table 2. ANOVA analysis and Tukey's test on different parts in the stem of *D. thyrsiflorum* from different age when flowers are dying.

Age	Base of stem	Middle of stem	Top of stem
1-year-old stem	0.06[b]	6.72[a]	7.35[a]
2- year-old stem	0.06[b]	0.01[c]	5.78[b]
3-year-old stem	0.16[a]	1.94[b]	0.45[c]
value of significant difference (*P*)	0.000	0.000	0.000

Significant difference tests: a>b>c.

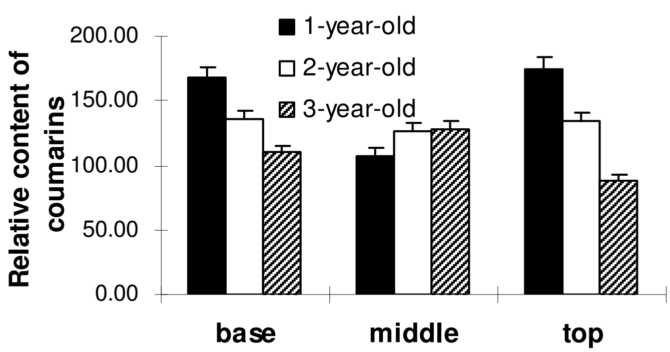

Figure 3. Dynamic changes of coumarins in different stem parts of *D. thyrsiflorum* from different age when flowers are blooming.

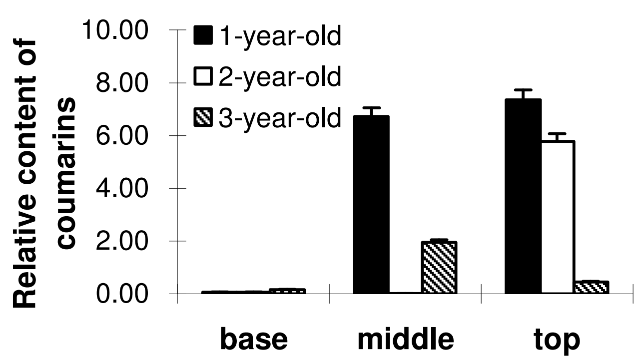

Figure 4. Dynamic changes of coumarins in different stem parts of *Dendrobium thyrsiflorum* from different age when flowers are dying.

plants by now. It has widened histochemical studies field. Today, it has been under-use as a methodology in phar-

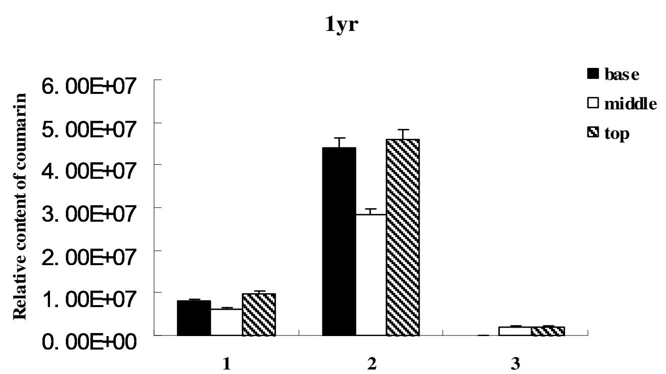

Figure 5. Dynamic change of coumarins in different parts of 1 year old stem of *D. thyrsiflorum* from different developmental stage (1: vegetative growth; 2: flowers blooming; 3: flowers dying).

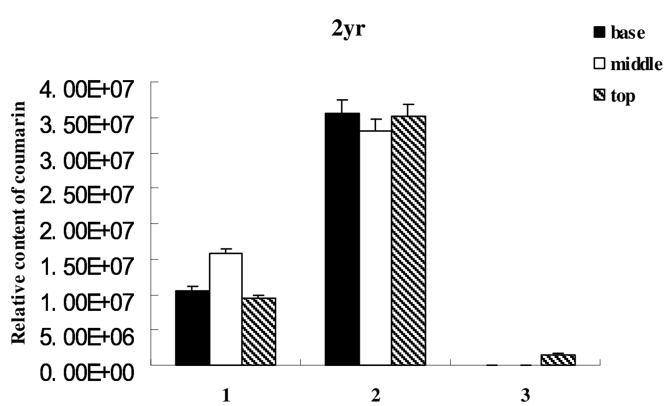

Figure 6. Dynamic change of coumarins in different parts of 2 year old stem of *D. thyrsiflorum* from different developmental stage (1: vegetative growth; 2: flowers blooming; 3: flowers dying).

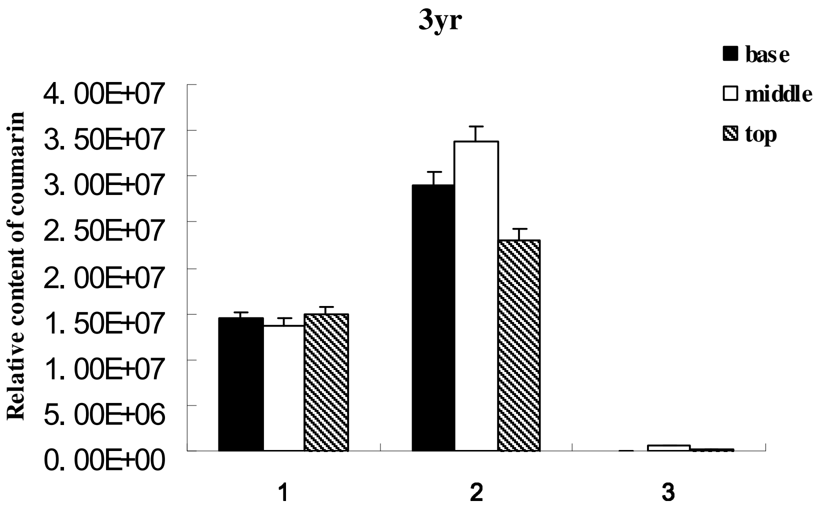

Figure 7. Dynamic change of coumarins in different parts of 3 year old stem of *D. thyrsiflorum* from different developmental stage (1: vegetative growth; 2: flowers blooming; 3: flowers dying).

Table 3. ANOVA analysis result of total fluorescence intensity of *D. thyrsiflorum* from different developmental phase.

Age	Stem parts	Total pixel intensity of coumarins			Significant value
		Vegetative growth stage	Flowers blooming stage	Flowers dying stage	
1-year old	Base	8.14 E + 06^{b}	4.42 E + 07^{a}	1.64 E + 04^{c}	***
	Medium	6.15 E + 06^{b}	2.82 E + 07^{a}	2.05 E + 06^{b}	***
	Top	9.90 E + 06^{b}	4.60 E + 07^{a}	2.02 E + 06^{c}	***
2-year old	Base	1.05 E + 07^{b}	3.56 E + 07^{a}	1.74 E + 04^{c}	***
	Medium	1.57 E + 07^{b}	3.31 E + 07^{a}	1.93 E + 03^{c}	***
	Top	9.37 E + 06^{b}	3.51 E + 07^{a}	1.57 E + 06^{c}	***
3-year old	Base	1.44 E + 07^{b}	2.90 E + 07^{a}	4.28 E + 04^{c}	***
	Medium	1.37 E + 07^{b}	3.37 E + 07^{a}	5.23 E + 05^{c}	***
	Top	1.50 E + 07^{b}	2.31 E + 07^{a}	1.18 E + 05^{c}	***

maceutical and other related research (Shotton and White, 1989; King et al., 1994; Tanji et al., 1999; Perez-de-Luque et al., 2006; Shenoy et al., 2006; Wang, 2006). This methodology is suitable not only for *D. thyrsiflorum* but for other original species of traditional Chinese medicine.

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