Identification of Ages and Determination of Paeoniflorin in Roots of \textit{Paeonia lactiflora} Pall. From Four Producing Areas Based on Growth Rings

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**KEY WORDS** \textit{Paeonia lactiflora} Pall.; \textit{Paeoniae radix} Alba; age identification; growth ring; annual ring; root; paeoniflorin

**ABSTRACT** Growth rings were used to determine the root age of medicinal \textit{Paeonia lactiflora} from four producing areas, and their corresponding paeoniflorin content were measured based on the identification of ages. Different \textit{P. lactiflora} root samples of different ages were collected from the four major growing areas in China: Bozhou, Anhui Province; Pan'an, Zhejiang Province; Zhongjiang, Sichuan Province; and Heze, Shandong Province. The relationship between the number of growth rings and age was analyzed using hand sections and paraffin sections. The paeoniflorin content in the roots of different \textit{P. lactiflora} cultivars from different growing areas was measured using high-performance liquid chromatography (HPLC). The growth rings in the \textit{P. lactiflora} roots were consistent with the age of the plant from Heze, Zhongjiang, Pan'an, whereas that for the \textit{P. lactiflora} from Bozhou was one less than the age of the plant. The HPLC results show that the paeoniflorin content was highest in \textit{P. lactiflora} ‘Baihuachuanshaoyao,’ followed by ‘Baihuahangshaoyao,’ ‘Honghuachuanshaoyao,’ and ‘Honghuahangshaoyao,’ ‘Bozhoushaoyao’ had the lowest levels of paeoniflorin. With increasing age, the paeoniflorin in the roots of the different \textit{P. lactiflora} cultivars slowly declined or remained the same. In summary, the age of the roots of \textit{P. lactiflora} from different growing areas can be determined using growth rings. The paeoniflorin content in the roots of \textit{P. lactiflora} is correlated with cultivar and it was slowly declined with increasing age. Microsc. Res. Tech. 75:1191–1196, 2012. © 2012 Wiley Periodicals, Inc.

**INTRODUCTION**

The age of many trees can be judged using their annual rings, which play an important role in the past climate change and mechanism (Esper et al., 2002). In recent years, the growth ring phenomenon was also observed in roots of some dicotyledonous perennial herbs, known as the “herbchronology” (Dietz et al., 1997). Currently, herbchronology has received increasing attention in studies on climate change and disturbance ecology (Dietz and Fattorini, 2002; Dietz et al., 2004; Schweingruber and Poschlod, 2005; Perkins et al., 2006). Growth rings have been reported in the roots of perennial forbs in the Duolun Grassland, Inner Mongolia, China (Liu and Zhang, 2007), but these studies are limited to the field of ecology, and the growth rings of Chinese medicinal plants have been rarely reported. Nearly 300 species of perennial herb roots are included in the Chinese Pharmacopoeia. The accumulation of secondary metabolites in medicinal plant roots is correlated with growth age (Soldati and Tanaka, 1984). Therefore, determining the age of herbaceous plant roots to investigate the secondary metabolite accumulation is important in root medicine. However, different from wild plants, some commonly used Traditional Chinese Medicines are distributed in different climatic zones and different altitudes after the plants were first introduced, and the cultivation methods are also different. These conditions make the growth ring phenomenon more complex in some perennial herbs.

\textit{Paeonia lactiflora} Pall. is an herbaceous perennial, and its roots have been used in medically for at least 2000 years in China (Peng and Wang, 2007). The Chinese Pharmacopoeia (Pharmacopoeia Commission of the People’s Republic of China, 2010) defines \textit{Paeonae radix} Alba as the roots of \textit{P. lactiflora} that have been peeled, boiled, and dried \textit{Paeoniae radix} Alba is derived from different cultivars of \textit{P. lactiflora}. Currently there are six \textit{P. lactiflora} cultivars in the four major growing areas: Bozhou, Anhui Province; Pan'an, Zhejiang Province; Zhongjiang, Sichuan Province; and Heze, Shandong Province (Zha et al., 2011a). The breeding and cultivation methods of \textit{P. lactiflora} vary among the four areas. Cultivation of \textit{P. lactiflora} usually lasts 3–6 years, and it can be harvested after 3–6 years of cultivation(Zha et al., 2011b).The active ingredients in the roots of \textit{P. lactiflora} is total glucosides of paeony (TGP)

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Received 7 January 2012; accepted in revised form 29 February 2012
Contract grant sponsor: National Natural Science Foundation of China; Contract grant number: 30901973
DOI 10.1002/jemt.22048
Published online 17 April 2012 in Wiley Online Library (wileyonlinelibrary.com).
(Wu et al., 2009), which has sedative, analgesic, anti-inflammatory, hepatoprotective properties, as well as other pharmacologic activities. Paeoniflorin accounts for over 90% of TGP. A number of scholars have conducted various studies to measure paeoniflorin content in _Paeoniae radix_ Alba, including determining the paeoniflorin content in _P. lactiflora_ of different ages from different growing areas, but their results are inconsistent (Hong et al., 2003; Hu and Liang, 2009; Li et al., 2005; Meng and Jiang, 2008; Wang and Chen, 2007; Wu et al., 2006). These inconsistencies may be attributed to the inability to clearly identify the age of _P. lactiflora_ roots. Therefore, determining the root age of _P. lactiflora_ can be a reasonable assessment of the relationship between paeoniflorin content and age of _P. lactiflora_ roots.

The objective of this article are as follow: (1) to report the relationship between growth ring in roots of _P. lactiflora_ and growing years, and (2) based on the age identification, elucidate the correlation of _P. lactiflora_ root age and paeoniflorin accumulation in different growing areas.

**MATERIALS AND METHODS**

The _P. lactiflora_ specimens from Bozhou, Anhui Province; Pan’an, Zhejiang Province; Zhongjiang, Sichuan Province; and Heze, Shandong Province (Table 1).

**Paraffin Sectioning Method**

Fresh roots of _P. lactiflora_ were harvested. The samples were collected 1 cm from the root head, fixed with FAA solution (70% ethanol:formaldehyde:acetic acid = 90:5:5), vacuumed, and dehydrated using different alcohol concentrations. Then, the sections were embedded in paraffin. The prepared sample was baked on a Leica H11220 flattening table, and sectioned with a Leica RM2265 rotary microtome to slice thicknesses of 10–15 μm. The samples were then baked for more than 24 h, deparaffinized, stained with safranin-fast green solution, observed and photographed under an OLYMPUS stereo microscope.

**Hand Sectioning Method**

Samples were collected from 1 cm from the root head of fresh _P. lactiflora_ roots, at a thickness of about 1 mm. A drop of phloroglucinol–hydrochloric acid reagent was used to develop color, and a scanner was used to take images.

**Statistics of Growth Ring in Roots of _P. lactiflora_**

One centimeter long samples were collected from all roots in 1- to 4-years old _P. lactiflora_ root system from four major growing areas. The numbers of growth rings in all roots from the root system were observed under a microscope to analyze the number of growth rings in the root system from the four major growing areas.

**Experimental Determination of Paeoniflorin Content**

_P. lactiflora_ processing at the growth area requires peeling and boiling in water. The sequences and time of peeling and water boiling are different in the various growing areas, and these differences affected the results of the measurement of paeoniflorin content in _P. lactiflora_ (Si and Gu, 2004). To reduce the differences, all samples were processed by direct drying method (i.e., no peeling or water boiling). Dried root samples of _P. lactiflora_ were crushed into powder, and screened using a 60-mesh sieve. The equipment used included the following: Agilent 1100 HPLC, HSS120 digital ultrasonic cleaner, chromatography column, and quartz sub-boiling high purity water extractor. A mobile phase of acetonitrile:water (18:82) was used at a flow rate of 1.0 mL min<sup>−1</sup>, a detection wavelength of 230 nm, column temperature of 25°C, and an injection volume of 10 μL; the theoretical plate number was not <2,000 as calculated for paeoniflorin.

**Preparation of Standard Solutions.** An appropriate amount of paeoniflorin standard was accurately weighed and diluted in methanol to 60 μg mL<sup>−1</sup>

**Preparation of the Sample Solution.** Dry _P. lactiflora_ root powder was accurately weighed, placed in a 50 mL volumetric flask, diluted with 35 mL of 50% ethanol, ultrasonicated for 30 min, cooled, supplemented with 50% ethanol to the mark, shaken to mix thoroughly, and filtered to acquire a standard solution.

**Linear Relationship Investigation.** The standard solution was accurately injected into a liquid chromatograph at 2, 5, 10, 15, 20, and 20 μL. A standard curve was made using the peak area as the y-axis and standard volume (μg) as the x-axis. The obtained regression equation was linear: Y = 99.104X + 165.81, r = 0.9991. The results show that paeoniflorin standard curve has good linearity at concentrations ranging from 0.4509 to 2.2545 μg.

**TABLE 1. Experimental _P. lactiflora_ samples**

<table>
<thead>
<tr>
<th>Growth area</th>
<th>Harvest place</th>
<th>Taxon</th>
<th>Harvest time</th>
<th>Age (year)/number of plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bozhou City, Anhui Province</td>
<td>Shihali Village, Shijuli Village</td>
<td><em>Paeonia lactiflora</em></td>
<td>2009–10</td>
<td>1/8, 2/4, 3/6, 4/6, 5/4</td>
</tr>
<tr>
<td>Heze City, Shandong Province</td>
<td>Huatuo Village, Xiaodu Village, Zhu Village, Daizhu Village</td>
<td><em>Bozhoushaoyao</em></td>
<td>2009–10</td>
<td>1/10, 2/5, 3/4, 4/4</td>
</tr>
<tr>
<td>Pan’an County, Zhejiang Province</td>
<td>Xinwo Village</td>
<td><em>Paeonia lactiflora</em></td>
<td>2010–5</td>
<td>1/9, 2/8, 3/6, 4/6, 5/5</td>
</tr>
<tr>
<td>Zhongjiang County, Sichuan Province</td>
<td>Jifeng Village</td>
<td><em>Baihuahangshaoyao</em>; <em>Paonia lactiflora</em></td>
<td>2010–5</td>
<td>1/9, 2/8, 3/4, 4/7</td>
</tr>
</tbody>
</table>

*Microscopy Research and Technique*
Precision Analysis. Exactly 10 μL of the same paeoniflorin standard solution was injected six times each time, which indicated an RSD of 0.45% ($n = 6$).

Repeatability Analysis. The same paeoniflorin sample solution was accurately taken, processed as described for sample solution preparation method, and injected as above chromatographic conditions. The average paeoniflorin content in the peony root was 6.24%, with RSD = 0.76% ($n = 6$).

Measurement Method. A 10 μL standard solution and 10 μL sample solution were accurately taken and injected into liquid chromatograph for measurement.

RESULTS

Growth Rings in Roots of *P. lactiflora* From Heze City

There is only one cultivar, *P. lactiflora* 'Hezeshaoyao,' in Heze, Shangdong Province (Zha et al., 2011a) and it
is reproduced using seeds. The root system has an obvious main root, adventitious roots, and lateral roots (Fig. 1C). The paraffin sections of the *P. lactiflora* ‘Hezeshaoyao’ of different ages revealed clear growth rings in the xylem of the secondary roots, and the number of growth rings in main root was consistent with an age of 1–5 years (Figs. 1B, 1E, 1F, 1G, and 1H). The hand section stained with phloroglucinol HCl also revealed clear visible growth rings, and the results are consistent with the paraffin sections (Figs. 1A and 1D). Only one main root was present in the root system of *P. lactiflora* and the number of roots with the maximal number of growth rings in the entire system was usually 1. The lateral roots and adventitious roots develop later, and the number of growth ring was correspondingly lower. However, for some seeds, after the main root germinates, the rhizomes will also give rise to more adventitious roots in the same year, and the number of growth rings in the adventitious roots was same as that in the main root, leading to more roots with same maximal number of growth rings in the root system.

**Growth Ring in Roots of *P. lactiflora* From Bozhou City**

There is only one cultivar, *P. lactiflora* ‘Bozhoushaoyao’ in Bozhou, Anhui Province (Zha et al., 2011a), and it is propagated using rhizomes. All roots are removed when rhizomes are used for cultivation. Thus, all roots in the root system develop from adventitious roots. The number of growth rings in root systems that have grown for 1 year was zero. As root system grew, a certain number of adventitious roots developed each year. In biennial root systems, some roots grow for 2 years (the number of growth ring is 1) and 1 year (the number of growth rings is 0). Therefore, in 3-year-old root systems, the roots have 2, 1, and 0 growth rings. In 4-year-old root systems, the roots have 3, 2, 1, and 0 growth rings. Thus, the age of *P. lactiflora* ‘Bozhoushaoyao’ root is equal to its growth ring number plus 1.

**Growth Rings in Roots of *P. lactiflora* From Zhongjiang County, Sichuan Province**

There are two cultivars, *P. lactiflora* ‘Honghuachuanshaoyao’ and *P. lactiflora* ‘Baihuachuanshaoyao’ in Zhongjiang, Sichuan Province (Zha et al., 2011a). Rhizome propagation is used to cultivate *P. lactiflora* ‘Bozhoushaoyao’ wherein roots are removed when cultivated. However, the number of growth rings in the root systems of *P. lactiflora* ‘Honghuachuanshaoyao’ and ‘Baihuachuanshaoyao’ are different from *P. lactiflora* ‘Bozhoushaoyao’. *P. lactiflora* ‘Honghuachuanshaoyao’ and ‘Baihuachuanshaoyao’ grown for 1 year have one ring. The number of growth rings increased by one each year as the root system developed. That is, the...
growth rings of the *P. lactiflora* from Zhongjiang are annual.

**Growth Ring in Roots of *P. lactiflora* From Pan'an County, Zhejiang Province**

There are two cultivars, *P. lactiflora* 'Honghuahangshaoyao' and *P. lactiflora* 'Baihuahangshaoyao' in Pan'an, Zhejiang Province (Zha et al., 2011a). Both cultivars are cultivated using rhizome propagation, and some roots are left on the rhizome when cultivated. The growth ring number and age were consistent in most roots in the root system of *P. lactiflora* cultivars from Pan'an and the growth rings are annual. However, the number of growth rings in a few roots can exceed their ages. These roots are left during cultivation, and are grown for 1–3 years. Figure 2A was the statistical analysis of the growth rings in the root system of *P. lactiflora* of different ages in Bozhou, B was in Heze, C was in Zhongjiang, and D was in Pan'an.

**Paeoniflorin Accumulation Dynamics in *P. lactiflora* Roots With Different Growing Years From Different Producing Areas**

Based on the age determination method, the ages of the *P. lactiflora* roots were determined at each growth area and measured the paeoniflorin content in samples with different growing years from Bozhou, Pan'an, and Zhongjiang (Fig. 3). The results show that the paeoniflorin content in the roots of *P. lactiflora* 'Bozhoushaoyao' did not change significantly with increasing age, but it declined significantly in other peony cultivars. The paeoniflorin content in the five different cultivars were significantly different, with the maximal level in roots of 'Baihuachuanshaoyao,' followed by 'Baihuahangshaoyao,' 'Honghuachuanshaoyao,' 'Honghuahangshaoyao,' and 'Bozhoushaoyao.'

**DISCUSSION**

The different ages of roots of *P. lactiflora* at four major growing areas were identified through the use of paraffin sections, hand sections, and microscopic observation methods. The growth ring phenomenon was observed in roots of *P. lactiflora* from all the four major growing areas for the first time, and growth rings can be used to identify age of *P. lactiflora*. Based on the age identification, the paeoniflorin content was measured in *P. lactiflora* of different ages at different growing areas. This addresses previous deficiencies in measuring paeoniflorin content in *P. lactiflora* of different ages.

*P. lactiflora* is a perennial herb. It germinates and flowers in spring, and withers in winter (Hong et al., 2001). Most roots of *P. lactiflora* grow to 20–25 cm below the ground surface and are sensitive to surface temperature. Among the four major *P. lactiflora* growing areas, Bozhou and Heze are located in a warm temperate region, and Zhongjiang and Pan’an are located in a subtropical region (Zha et al., 2011a,b). The seasonal climate significantly changes in all four areas and the ground temperature regularly changes with the climate, so the vessels in the *P. lactiflora* roots also change regularly to form growth rings. The experimental results show that the roots from the six cultivars in the four growing areas all had growth rings. The number of growth rings in 'Bozhoushaoyao' was 1 less than the actual age, whereas those from the other three growing areas, regardless of the method propagation were consistent with the actual age, i.e., the growth rings are annual. Through this experiments, especially the hand sections and the phloroglucinol color development method, the age of *P. lactiflora* from the four major growing areas can be quickly determined.

The active ingredient in *P. lactiflora* roots is TGP (Wu et al., 2009), which has sedative, analgesic, anti-inflammatory, hepatoprotective activities, as well as other pharmacologic properties. Paeoniflorin accounts for over 90% of TGP. Chinese Pharmacopoeia Commission of the People's Republic of China, 2010 used paeoniflorin content as an indicator to monitor the quality of *Paeoniae radix* Alba. A number of scholars have conducted various studies to measure paeoniflorin content in peony, including determining the paeoniflorin content in *P. lactiflora* of different ages from different growing areas, but their results are inconsistent (Hong et al., 2003; Hu and Liang, 2009; Li et al., 2005; Meng and Jiang, 2008; Wang and Chen, 2007; Wu et al., 2006). These inconsistencies may be attributed to the inability to clearly identify the age of *P. lactiflora* roots. Considering the *P. lactiflora* root system has roots of different ages, the duration of cultivation of the root system does not represent the age of each root. The methods for *P. lactiflora* processing are different among the various growing areas (Si and Gu, 2004). In this experiment, based on the identification of the root age of *P. lactiflora*, a uniform processing method was used to measure the paeoniflorin content in *P. lactiflora* of different ages from the different growing areas. The results show that the different cultivars had significantly different paeoniflorin content, with the highest level in 'Baihuachuanshaoyao,' followed by 'Baihuahangshaoyao,' 'Honghuachuanshaoyao,' 'Honghuahangshaoyao,' 'Baihuachuanshaoyao,' and 'Bozhoushaoyao.'
ACKNOWLEDGMENTS

The authors acknowledge the guidance and support of Prof. Xiaomei Xie and Prof. Ling Zhang from the Pharmacy School of Anhui University of Traditional Chinese Medicine during the experiment.

REFERENCES

Wu XZ, Wang MF, Yang SY, Lang YH. 2006. Determine the content of paeoniflorin which in the different parts and in the different growth apprenticeships in Radix Paeoniae Alba. Zhong Guo Xian Dai Ying Yong Yang Yao Xue Za Zhi 4:291–293.

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