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RECENT STUDY ON PLANT-SOIL INTERACTIONS IN CHINA - PART I

Effects of cultivation years on effective constituent content of *Fritillaria pallidiflora* Schrenk

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Abstract

Fritillaria pallidiflora Schrenk has been treasured in traditional classic medicine as an antitussive, antiasthmatic and expectorant for hundreds of years. With gradually decreasing wild *F. pallidiflora* resources, the herb can no longer satisfy the demand. Artificial cultivation is one of the most effective ways to solve the contradiction between supply and demand in the medicinal material market. During the growth of Rhizomes medicinal plants, root biomass and active ingredient content showed dynamic accumulated variation with increasing cultivation years. Up to now, hardly any attempts have been made to investigate the relationship between quality and cultivation years of *F. pallidiflora*. Therefore, in this paper, we determined the optimum harvesting time by comparing biomass and biological characteristics of *F. pallidiflora* at different cultivation times. High-performance liquid chromatography with evaporative light scattering detection and phenol-sulfuric acid visible spectrophotometry was performed to determine imperialine and polysaccharide content of *F. pallidiflora* bulbs. From year 1 to 6 of cultivation, we observed an upward trend in plant height, diameter and dry weight of *F. pallidiflora*, while water content decreased. Plant height and dry weight increased remarkably during the fourth year of cultivation. The content of imperialine and polysaccharide of *F. pallidiflora* bulbs, on the other hand, showed an upward trend from year 1 to 3, after which it decreased from year 3 to 6. By comparing plant growth, biomass development and the accumulation of imperialine and polysaccharide, the best harvesting time of *F. pallidiflora* was determined to be after 4 years of cultivation. Our results showed that it is possible to establish a “safe, effective, stable and controllable” production process, which could play an important role in achieving sustainable utilization of *F. pallidiflora* resources.

Keywords: *Fritillaria pallidiflora* Schrenk, cultivation years, growth and development, imperialine, polysaccharide, HPLC

Introduction

Currently in China, more and more wild medical plants are being cultivated. Such measures play an important role in the conservation of biodiversity (Ishtiaq et al. 2010; Yang et al. 2010; Tisdell 2011; Pradhan & Badola 2012). The dry bulbs of *Fritillaria pallidiflora* Schrenk, a perennial herb belonging to the *Fritillaria* genus of the Liliaceae family, have been used in traditional Chinese medicine (TCM) for more than 2000 years as an antitussive, antiasthmatic and expectorant medicinal herb, called “Beimu” in Chinese (Shang & Liu 1995). It is mainly distributed along the Ili River basin of Xinjiang, especially in Xinjiang Yili, Altay, Tacheng and other places. Due

to the increased market demand, wild *F. pallidiflora* is in short supply. Long-term excessive digging has increasingly exhausted wild resources, and as a result, the species has been classified as a level-3-protected medicinal plant. In recent years, with continuous research and development of new drugs derived from *F. pallidiflora*, its demand has increased even further.

Alkaloids and saponins are the main effective ingredients of the *Fritillaria* genus, including verticine, verticinone, imperialine and alkaloid glycoside (Xu et al. 1990; Li et al. 1991; Rahman et al. 1998; Li et al. 2004). Among other pharmacological effects, alkaloids from *Fritillaria* have been shown to relieve asthma, reduce phlegm, lower blood

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pressure and relax muscle spasms. Imperialine is the most abundant alkaloid of *F. pallidiflora*, with the highest content present in the bulb, considered the storage organ of imperialine as secondary substances (Sha et al. 1988). Pharmacological research showed that the main effective ingredients in *F. pallidiflora* are imperialine and its glycosides, which have significant relaxation and anticholinergic activity in isolated rat trachea (Chen et al. 2011) and bronchus (Rahman et al. 1998).

Previous research showed that the chemical constituents of wild plants change during artificial cultivation (Rühl et al. 2011; Donnini et al. 2013; Selbmann et al. 2013). Polysaccharides are one of the major bioactive substances of Chinese herbal medicine, equipped with various pharmacological activities such as immunoregulation, tumor suppression, lowering of blood sugar and fat and defence against infection (Fang 1994; Li et al. 1997; Gu et al. 1999; Liu et al. 2002; Kardosova et al. 2006; Jung-Bum et al. 2010). *Fritillaria ussuriensis* Maxim polysaccharide FUP-1 was shown to have free radical scavenging activity and might function in the delay of aging, possibly through mechanisms related to the activation of relevant antioxidant enzymes and to the inhibition of lipid peroxidation (Liu et al. 2011a,b). Plant polysaccharides are commonly found in plants and have no cytotoxicity effects, which may result smaller side effects. Therefore, the study on the medical use of plant polysaccharides has become a hot field.

Harvesting time plays a vital role in ensuring compound quality as well as in protecting and expanding drug resources. Research has shown that the content of total saponins and flavonoids in *Glycyrrhiza uralensis* reached a maximum after 3 years of culture, after which it slowly decreased with increasing cultivation years. Therefore, 3-year-old cultivated *G. uralensis* plants were chosen as an optimal source for harvesting and processing (Wang et al. 1991). The content of saponins of *Rhizoma anemarrhenae* also increased with cultivation years, with no significant difference in content during spring and autumn. The optimal harvest time was determined to be after 3 years of cultivation (Han et al. 1993). Content of paeoniflorin and total glycosides in 4-year old cultivated *Paeonia lactiflora* Pall plants, the basis of the Chinese herb Radix Paeoniae Alba, are highest between September and October, is thus considered as the best harvesting time (Jin et al. 2010). The harvest time of *Liriope muscari* was shown to be optimal in May; when the root expanded, the biomass production sharply increased and the level of 25(R,S) ruscogenin 1-O- $[\beta$ -D-glucopyranosyl (1 \rightarrow 2)] $[\beta$ -D-xylopyranosyl (1 \rightarrow 3)] β -D-fucopyranoside

was highest (Hu et al. 2010). In case of *P. felterrae*, the best harvesting period was shown to be at the early fruit stage (Ning et al. 2012). Thus, timely harvesting plays an important role in ensuring the quality of Chinese medicinal materials.

F. pallidiflora Schrenk has been used as in TCM to cure cough, sputum and asthma. However, with the gradual decrease in wild *F. pallidiflora* resources, the herb could no longer satisfy people's demand. Artificial cultivation would be one of the most effective ways to solve the contradiction between supply and demand of this species in the medicinal material market. Up to now, hardly any research has been carried out on the relationship between quality and cultivation years of *F. pallidiflora*. Therefore, the aim of our research was to study its growth and development and to determine the optimum harvest time by measuring biomass and the accumulation of imperialine and bioactive polysaccharides during cultivation years. We hope we can contribute to an expansion in the use of medicinal material resources obtained from artificially grown plants, with the purpose of establishing a "safe, effective, stable and controllable" production process, which will also be of great significance in achieving the sustainable utilization *F. pallidiflora* resources.

Materials and methods

Study site

F. pallidiflora Schrenk plants of different ages (between 1 and 6 years of cultivation) were collected during the dormancy period at Wulasitai Village, Nilka County, Yining City, Xinjiang Province of China (altitude: 1438 m; 43°49'N, 83°07'E) in June 2011 and identified by Yu-Huai Cheng at the Shihezi University.

Measurements of biomass and active constituent content

Biomass determination of *F. pallidiflora* Schrenk was performed according to the method described by Zhou (2000). Determination of imperialine content was carried out following methods in China pharmacopoeia (2010 edition), and the content of polysaccharide was determined as described by Wang et al. (2005).

Data analysis

Statistical data analysis was performed using the SPSS software package for Windows 13.0 (SPSS, Chicago, IL, USA).

Results

Characteristics of cultivated *F. pallidiflora* Schrenk plants

F. pallidiflora plant height increased with cultivation years, reaching a maximum of 41.7 cm at year 6 (Figure 1). Relative plant height ratio from year 1 to 6 was 1:1.1:1.6:5:7:8.7, showing that plant height changed only slightly (3.0 cm) over the first 2 years (year 1–3). After the third year, plants started to grow rapidly, with an increase in plant height of 16.4 cm (year 3–4).

Figure 2 shows that the bulb diameter of *F. pallidiflora* Schrenk increased from minimum of 0.715 cm at year 1 to the maximum of 3.221 cm at the sixth year. The relative bulb diameter from year 1 to 6 was 1:1.8:2.4:3.2:3.9:4.5. These results indicate that cultivation years and bulb diameter are linearly correlated.

Figure 3 shows that water content of *F. pallidiflora* remained above 70% for the whole cultivation period, with a maximum value of 77.53% in the first year and a minimum of 72.13% in the sixth year. The relative water content ratio of *F. pallidiflora* bulbs from year 1 to 6 was 1:0.95:0.95:0.94:0.94:0.93. The water content of *F. pallidiflora* cultivated between 3 year and 5 years was significantly different from the others, with a reduction of about 4%. From year 2 to 6, the bulb water content continued to decrease, although at a slower rate.

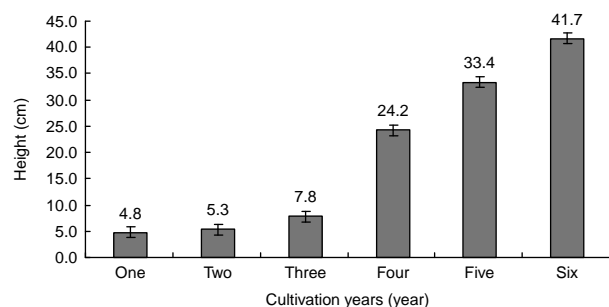


Figure 1. Height of cultivated *Friillaria pallidiflora* Schrenk plants.

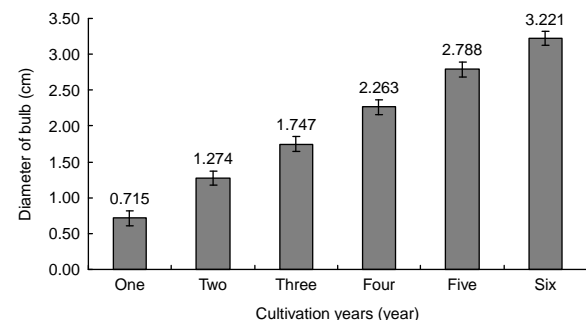


Figure 2. Bulb diameter of cultivated *F. pallidiflora* Schrenk.

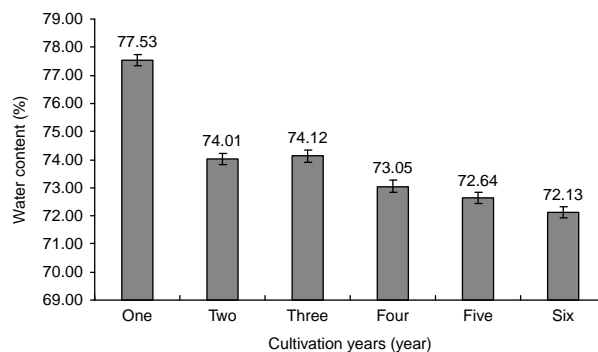


Figure 3. Water content of cultivated *F. pallidiflora* Schrenk.

Figure 4 shows that biomass of *F. pallidiflora* bulbs increased slightly with increasing cultivation years, reaching a maximum of 1.723 g at year 6, while the minimum value at year 1 was only 0.017 g. The relative biomass ratio of the *F. pallidiflora* bulbs from year 1 to 6 was 1:4.9:21.8:72.6:92:99.6. Biomass increased significantly at the beginning of the third year, reaching a bulb dry weight value of 1.2 g at year 4. The results show that bulbs of cultivated *F. pallidiflora* grew fastest from year 3 to 4.

To determine the imperialine content of *F. pallidiflora*, we performed high-performance liquid chromatography with evaporative light scattering detection (HPLC-ELSD) (Figures 5 and 6). Imperialine content of *F. pallidiflora* increased approximately six-fold during the first 3 years, from $52.01 \mu\text{g g}^{-1}$ at year 1 up to a maximum value of $301.37 \mu\text{g g}^{-1}$ after year 3 of cultivation (Table I). Values decreased only slightly during the next 2 years of cultivation, but showed a sharp drop of 65% in the sixth year.

Table II shows that polysaccharide content of *F. pallidiflora* bulbs was also influenced by cultivation time, with an increase during the first 3 years to a maximum of 2.82%. At the fourth year, however, values dropped to below the level of year 1 and continued to decrease during the following years. This pattern may be related to the growth and development of *F. pallidiflora* plants.

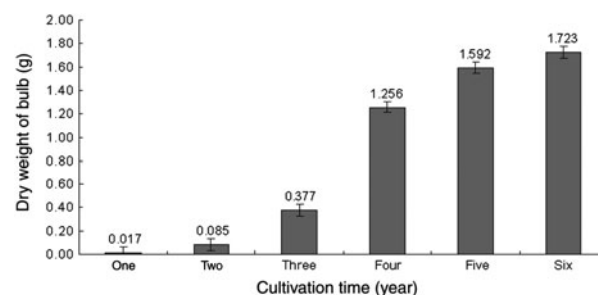


Figure 4. Bulb biomass of cultivated *F. pallidiflora* Schrenk.

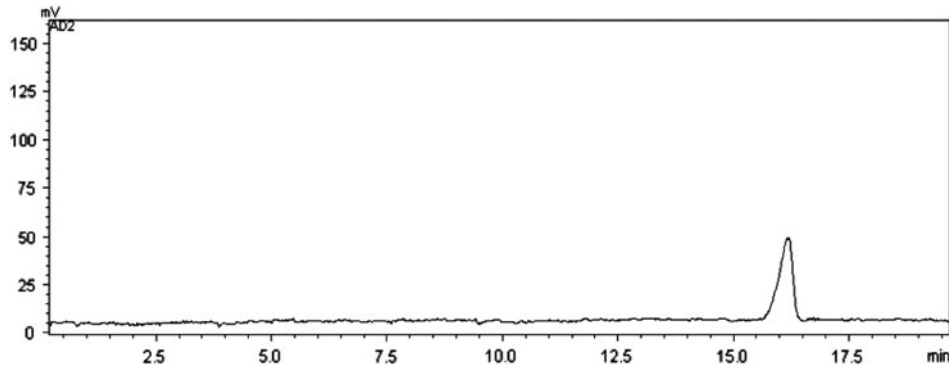


Figure 5. HPLC chromatogram of reference substance solution.

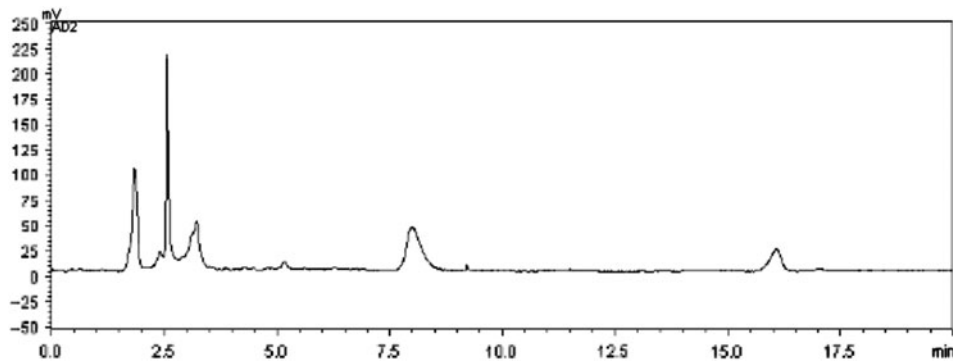


Figure 6. HPLC chromatogram of sample solution.

Table I. Imperialine content of cultivated *F. pallidiflora*.

Cultivation time (years)	Imperialine content ($\mu\text{g g}^{-1}$)	RSD (%)
1	52.01 ± 0.4629	0.89
2	129.73 ± 1.3232	1.02
3	301.37 ± 2.2523	0.74
4	296.48 ± 2.4608	0.83
5	293.79 ± 3.5549	1.21
6	104.73 ± 1.1730	1.12

Table II. Polysaccharide content of cultivated *F. pallidiflora* Schrenk.

Cultivation time (years)	Polysaccharides content (%)	RSD (%)
1	1.87 ± 0.0047	0.25
2	2.29 ± 0.0030	0.13
3	2.82 ± 0.0124	0.44
4	1.70 ± 0.0032	0.19
5	1.50 ± 0.0045	0.30
6	1.47 ± 0.0043	0.29

Discussion

Root biomass and content of active ingredients show dynamic accumulated variation with cultivation years in root medicinal plants (Stanisci et al. 2010; Manschadi et al. 2010; Abbasi et al. 2011; Giorio &

Nuzzo 2012; Mattana et al. 2012). The paeoniflorin content of cultivated *P. lactiflora* plants, for example, was positively correlated with cultivation years, but also varied during the season with highest levels found in summer. Huang et al. (2000) determined the optimum harvesting time with respect to active ingredients and medical traits to be after 3–4 years of cultivation, with a 6–7-month period for collecting material. *Baicalensis* cultivated for 1 year (root) or 3 years (caulis, leaf) was less suitable for medicinal purposes. The plant cultivated for 2 years (root), on the other hand, had a higher content of main active components with a higher quality, which could, therefore, replace the wild *Baicalensis* used for clinical applications (Yan et al. 2006). There were also notable differences in appearance traits, quality and GPS content among cultivated radix *Gentianae macrophyllae* samples of different ages, the best harvest time being after 4 years of cultivation (Chen et al. 2010). In a study by Li et al. (2011), it was shown that the content of main constituents in *Panax japonicus* varied greatly during cultivation, with a particularly apparent downward trend between year 3 and 4. The highest content was found in plants cultivated for 6 years, which is thus the optimum harvesting year. Another study showed that triptolide content of *Tripterygium wilfordii* varied greatly with

harvesting time, with the highest levels observed in December and the lowest in April. Furthermore, it was found that triptolide content declined with increasing root diameter (Ren et al. 2011).

F. pallidiflora schrenk presents distinct distribution characteristics of vertical zonality. Wild planting of *F. pallidiflora* can expand resources by replacing wild *F. pallidiflora* on the supply market, which is an effective way to protect wild *F. pallidiflora* populations and achieve a sustainable utilization of resources. Alkaloids and saponins are the main effective ingredients in *F. pallidiflora*, especially imperialine and imperialine-3 β -D-glucoside (Huang et al. 1990; Xu et al. 1990, 1993; Li et al. 2001). Total alkaloid content of *F. pallidiflora* was related to collecting time. A higher content was found at the pregnant bud and anthesis stages, while a lower content was observed during the fruiting period and after seedling (Chinese Academy of Medical Sciences Institute 1979). *F. pallidiflora* should not be cultivated for too long as total alkaloid content decreased with increasing cultivation years. Bulb growth, on the other hand, showed the opposite trend. An optimum cultivation period of 4 years was established for medicinal herb processing (He 1988). In *F. taipaiensis*, the content of peimisine increased during the growing stage from year 2 to 3, while it decreased from year 4 to 6. The content of total alkaloids increased during the growing stage from year 2 to 4 of cultivation, while it decreased from year 5 to 6 (Wang et al. 2011). The most suitable harvest time of *F. taipaiensis* was determined to be after a 4-year growing stage. Different batches of *F. pallidiflora* also differed greatly in their imperialine and imperialine-3 β -D-glucoside content. Taking objective reasons, such as collecting and quality instability, into account, content limitation should not be less than 0.4% for imperialine-3 β -D-glucoside (C₂₇H₄₃NO₃), and 0.02% for imperialine (C₃₃H₅₃NO₈) (Xu et al. 1994; Li et al. 1997). In the present study, we established quality standards for *F. pallidiflora*.

Growth and development of *F. pallidiflora* Schrenk is closely related to years of cultivating, the species being a perennial herb. The presented study showed that during cultivation of *F. pallidiflora*, there is an upward trend in plant height, diameter and dry weight, while the water content dropped, in particular at year 2. Imperialine content of *F. pallidiflora* bulbs was determined by HPLC analysis. During the first years of cultivation, the imperialine content of *F. pallidiflora* showed a six-fold increase, from the 1-year minimum of 52.01 $\mu\text{g g}^{-1}$ to the maximum value of 301.37 $\mu\text{g g}^{-1}$ after 3 years. However, from the third year onwards, the levels decrease to about one-third of the maximum level at year 6. Analysis of water content of *F. pallidiflora*

bulbs showed that there was no obvious change in water content from year 2 to 6 of cultivation.

Polysaccharide is one of the most important active constituents of *Fritillaria*, possessing anti-aging properties. The water-soluble polysaccharide of *F. ussuriensis* Maxim, FUP-1, is a heteropolysaccharide of which the monosaccharide part is composed of xylose, glucose and galactose, with a molar ratio of 5:1:1 (Liu et al. 2011a,b). Jiang et al. (2011) obtained a polysaccharide yield of up to 7.43% using an extraction time of 2.5 h at 90°C and a solid-liquid ratio of 1:50. The polysaccharide content of *Umbroact fritillary* bulbs was higher than that of *Bulbus fritillariae* Thunbergil. In addition, different producing areas yielded *U. fritillary* bulbs with obvious differences in polysaccharide content, which may be related to soil characteristics and climatic conditions (Wang et al. 2009). In cultivated *F. pallidiflora*, polysaccharide content is related to cultivation period, showing a rise from year 1 to 3 and a decrease from year 3 to 6. The highest content of 2.82% was observed in plants cultivated for 3 years.

The production and accumulation of chemical compounds in plants is determined by their genetic constitution (Li and Li 1997; Copetta et al. 2011; Minggagud and Yang 2012; Jiménez and Sánchez-gómez 2012). *F. pallidiflora* Schrenk has been treasured in traditional classic medicine as an expectorant with antitussive and antiasthmatic properties for hundreds of years and has been well received by physicians and patients for its evidently therapeutic effects. With the development of the modern pharmaceutical industry, there has been a huge increase in the market demand for this plant, which goes beyond the supply range of the natural yield. The high yield of *F. pallidiflora* Schrenk with its higher content of alkaloids, its pest resistance and other advantages has laid a solid foundation for its cultivation. In this paper, we compared growth, biomass and dynamic accumulation of the effective components imperialine and polysaccharide of *F. pallidiflora* at different times of cultivation. According to active ingredient content, the best harvesting time is after 4 years of cultivation. Results of this study can provide a reference for the artificial cultivation of *Fritillary*, for quality control of medicinal material and for the full utilization and rational development of *F. pallidiflora* resources. Taking the complex chemical constituents of *F. pallidiflora* into account, the relationship between cultivation time and chemical composition needs to be further investigated.

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