

Growth and glycyrrhizin contents in *Glycyrrhiza uralensis* roots cultivated for four years in eastern Nei-Meng-gu of China

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(Received February 20, 2002. Accepted March, 13, 2002.)

Abstract

In China, the collection of wild *Glycyrrhiza* roots came to be restricted since there is a high possibility of inducing desertification. In order to compensate for the insufficiency of wild *Glycyrrhiza* resources with cultivated ones, the cultivation study of *Glycyrrhiza uralensis* has been carried out since 1998 and is still continuing. The cultivation field is located in the eastern region of Nei-Meng-gu, inner Mongolia, which is the habitat of *G. uralensis* and the major source of Dongbei-Gancao, Tohoku-Kanzo in Japanese. The seeds of *G. uralensis* were sown on May in 1998 and seedling roots were transplanted to the field the next May. The glycyrrhizin (GL)-content of 4-year-old lateral roots, which were newly grown from the transplanted seedling taproot, exceeded the Japanese Pharmacopoeia XIV standard (2.5% or more GL). This is the first report to examine GL-content of *G. uralensis* cultivated in northeast China related to cultivation years. However, since GL-content ($2.81 \pm 0.76\%$) of 4-year-old lateral roots was at a low level yet, further examinations on improvement of cultivation conditions are in progress to obtain suitable length and thickness of lateral roots, high GL-contents and adequate ingredients-composition similar to those of wild *Glycyrrhiza* roots currently in use.

Key words *Glycyrrhiza uralensis*, cultivation, glycyrrhizin, HPLC, licorice, Gancao.

Abbreviations Dongbei-Gancao (Tohoku-Kanzo), 東北甘草; GL, glycyrrhizin; Xibei-Gancao (Seihoku-Kanzo), 西北甘草.

Introduction

There are many kinds of traditional Chinese formulations in which *Glycyrrhizae Radix*, Kanzo in Japanese, serves as the principal and supporting drug. *Glycyrrhizae Radix*, Gancao in Chinese, has been prepared from the underground parts of wild *Glycyrrhiza* plants in northwest and northeast China. Recently in China, the collection of wild *Glycyrrhiza* plants came to be restricted since there is a possibility of inducing desertification. With the decline of resources of wild *Glycyrrhiza* in the traditional region, cultivated *Glycyrrhiza* plants became an additional resource and are anticipated from the standpoint of conserving the natural environment.

Our on-the-spot investigations on the producing dis-

trict of Gancao in China indicated that *Glycyrrhiza* plants have been cultivated from the early 1990's. It is possible that *Glycyrrhizae Radix* prepared from cultivated *Glycyrrhiza* roots might have different characteristics compared to that which is prepared from wild *Glycyrrhiza* plants currently in use.¹⁾ The Japanese Pharmacopoeia XIV (JP XIV) requires that *Glycyrrhizae Radix* for medical use should contain not less than 2.5% of glycyrrhizin (GL), one of the active principles of the drug.²⁾

Therefore, in order to compensate for the insufficiency of wild *Glycyrrhiza* resources with cultivated ones and also to examine how many years are necessary for *G. uralensis* plant to be cultivated to fit with the JP XIV standard, the cultivation study has been carried out in China since 1998. As compared to previous papers

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concerning *G. uralensis*³⁾ and *G. glabra*⁴⁾ cultivated in the "experimental plant gardens in Japan", the present study was designed to examine the characteristic of *G. uralensis* cultivated in the "field in China." The cultivation field is located in eastern Nei-Meng-gu, inner Mongolia, which is the habitat of *G. uralensis* and the major source of Dongbei-Gancao, Tohoku-Kanzo in Japanese. Our cultivation examination in Nei-Meng-gu has been still continuing and we report here the results obtained so far.

Materials and Methods

Cultivated Glycyrrhiza roots obtained from on-the-spot investigations on the producing district of Gancao in China : Three samples cultivated without transplanting in northwest (Xibei) region : 5-year-old taproots (sample A: collected in 2001 at Ding-bian, Shanxi province, sown in 1997), 6-year-old taproots (sample B: collected in 2000 at Hang-jin-qi, western region of Nei-Meng-gu, sown in 1995) and 11-year-old taproots (sample C: collected in 2001 at Jing-bian, Shanxi province, sown in 1991).

Three samples cultivated with transplanting in northeast (Dongbei) region: 2-year-old taproots (sample D: collected in 1999 at Weng-niu-te-qi, Nei-Meng-gu sown in 1998) and (sample E: collected in 1999 at Tong-yu, Jilin Province, sown in 1998) and 3-year-old taproots (sample F: collected in 1999 at Weng-niu-te-qi, Nei-Meng-gu, sown in 1997).

Cultivation of G. uralensis in Nei-Meng-gu : The present cultivation field is located in Yuan-bao-shan-qu, the suburbs of Chi-feng, eastern Nei-Meng-gu in China, 118° 97' east longitude and 42° 27' north latitude. The average annual rainfall of about 350 mm and the temperature changes between 23.5°C (average temp. in July) and -11.5°C (average temp. in January).

Artificial manure consisting of (NH₄)₂HPO₄ (1.5 kg / are), NH₄HCO₃ (2.3 kg / are) and KCl (1.5 kg / are) were applied before transplanting (on May in 1999) as basal dose. Urea (2.3 kg / are) was applied twice a year as additional fertilizer. Pesticide, Le-guo in Chinese preparation containing dimethoate: *O,O*-dimethyl *S*-methylcarbamoylmethyl phosphorodithioate, was applied twice a year to prevent insect plague.

Seeds collected in 1997 from wild *G. uralensis*

growing in Bao-tou, western Nei-Meng-gu were purchased at the market in An-guo, Hebei Province and sown on May in 1998. On May in 1999, the seedling taproots were cut to about 20-25 cm length and transplanted in the field by inclining about 10° and with a space of 40 cm between the roots (6-8 seedling roots / m²). The cultivated plants were identified as *G. uralensis* FISCH. with reference to the described report⁵⁾ by means of characteristic of undulate leaf-lets and falcate-fruits with prickly hairs of 3- and 4-year plants which came into flower in July and August. The voucher specimens have been deposited in Department of Pharmacognosy, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University.

On each sampling, the underground parts were dried in a dry-heat oven at 60°C for 3-5 days and the dry weight of each part was taken.

GL-content and other tests described in the JP XIV: Powdered sample (0.5g) prepared from whole dried taproot and lateral roots was extracted with 50% ethanol (70 ml) for 15 min under shaking 2 times and the solution was passed through a 0.45 μm filter as in the previous paper.¹⁾ GL-contents (% to dry weight of root) were measured by HPLC according to the procedures described in the JP XIV. The dilute ethanol-soluble extract (EtOH ext.) content and three kinds of tests (loss on drying, total ash, and acid-insoluble ash) were also measured by the procedures described in the JP XIV.

Statistics: Results were presented as the mean ± S.D. Student's *t*-test was performed for a comparison of the means using Excel 2000 (Microsoft). Probability (*p*) values less than 0.05 were considered significant.

Results and Discussion

Results of on-the-spot investigations on cultivated Glycyrrhiza roots in China (Fig. 1)

From our on-the-spot investigations in China on *Glycyrrhiza* roots cultivated in 1998 to 2001, *Glycyrrhiza* plants have been cultivated from the early 1990's in the northwest- and the middle of 1990's in the northeast-region. Details of cultivation conditions, especially transplanting of seedling roots varied among regions. In the northwest region of China (source of Xibei-Gancao, Seihoku-Kanzo in Japanese), *G. uralensis* is widely cultivated without transplanting the seedlings,

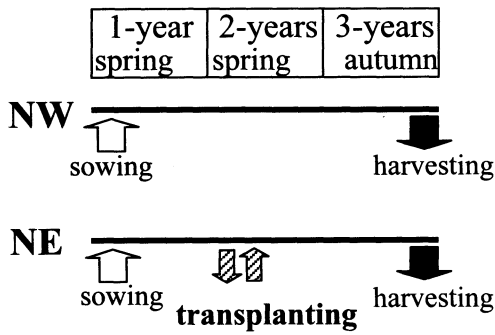


Fig. 1 General cultivation conditions of *G. uralensis* in the northwest (NW) and northeast (NE) region of China
 □: sowing; ▨: transplanting of seedling roots; ▣: harvesting.
 It is common in the northeast region (NE), Dongbei-Gancao producing districts, to sow the seeds in spring, and to dig up and transplant the seedlings the next spring. In this study, *G. uralensis* are cultivated with transplanting according to the conditions widely performed in the NE region.
 In some NE areas, the seeds of *G. uralensis* are sowed in spring and the seedlings roots are transplanted in the autumn of the same year and cultivated for 3 years.

whereas in the northeast region (source of Dongbei-Gancao, Tohoku-Kanzo in Japanese), seedling roots of *G. uralensis* are transplanted on the next spring of sowing (Fig. 1).

In Xibei-Gancao producing districts, Shanxi and Gansu province and western Nei-Meng-gu, GL-contents of the 5- and 6-year-cultivated samples (taproots) did not exceed the JP XIV standard (2.5% or more GL): 5-year-old sample A ($2.41 \pm 0.87\%$, n= 4) and 6-year-old sample B ($2.48 \pm 1.08\%$, n= 5). Although GL-content of 11-year-old sample C ($2.58 \pm 0.68\%$, n= 18) barely overstepped the JP XIV standard, no particular relationship between GL-contents and cultivation years is clearly noticed.

Wang *et al.*⁶⁾ reported that GL-contents of 3- and 4-year-old *G. uralensis* roots cultivated in the northwest region of China are about 8.18 and 8.53%, respectively. However, those of the 5- and 6-year-cultivated samples collected in our on-the-spot investigations were about 2.5%. The big difference between the values reported by the Chinese literature and those found by us was related to the different quantitative analysis method. Although we performed the determination in accordance to the HPLC method prescribed by the JP XIV, it is a pity that no quantitative analysis method is described in Chinese literature.⁶⁾

In Dongbei-Gancao producing districts, Jinlin province and eastern Nei-Meng-gu, 3-year-cultivated taproots have been sold in China and partly exported to Korea.

GL-contents of the 2- and 3-year-cultivated samples (taproots) collected also did not fit with the JP XIV standard: 2-year-old cultivated samples (taproots) also did not exceed the JP XIV standard: 2-year-old sample D ($1.35 \pm 0.58\%$, n= 7), sample E ($1.35 \pm 0.30\%$, n= 3) and 3-year-old sample F ($1.61 \pm 0.45\%$, n= 8). Since GL-contents showed increasing tendency with cultivation years, we tried to examine the relationship between GL-contents and cultivation years. For this purpose, we took field in the eastern region of Nei-Meng-gu to clarify how many years are necessary for *G. uralensis* plant to be cultivated in order to fit with the JP XIV standard.

Root Growth of G. uralensis cultivated in the eastern Nei-Meng-gu (Figs. 2-3)

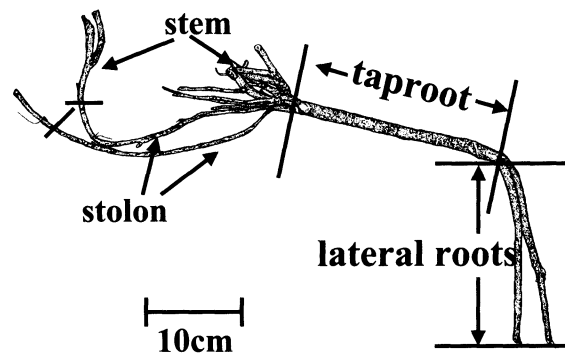


Fig. 2 Diagram of 4-year-old underground parts of *G. uralensis* taproot: the correspondence of transplanted seedling root, lateral roots: newly growing roots from tail of original taproot after transplanting. The samplings were made on August and October, 2000 for 3-year-old roots and June, August and October, 2001 for 4-year-old roots, respectively.

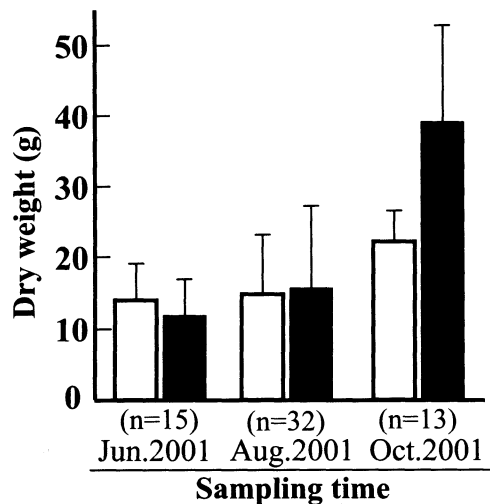


Fig. 3 Dry weight (g) of 4-year-old roots of *G. uralensis*
 Each value represents the mean \pm S.D.
 □: taproot, ■: lateral roots

In the contracted field (about 33 are), the 3- and 4-year-old plants grew to a height of 0.8-1.5 meters, which were taller than wild plants (0.4-0.6 m) growing in the surrounding wilderness. The sampling roots dug up from the field consisted of taproot which corresponds to transplanted seedling taproot, lateral roots and stolons (Fig. 2).

As shown in Fig. 3, the weight of taproots slightly increased during 4 years. On the other hand, lateral roots which grew from tail of taproot after transplanting were well developed and their weight (39.1 ± 13.6 g) harvested on October in 2001 exceeded that of taproot. The yield of 4-year-old fresh roots in October, 2001 was about 57 kg per are.

The number of lateral roots was not significantly different among cultivation years (2.7 ± 1.0 roots in Oct. 2000 and 2.6 ± 1.0 roots in Oct. 2001). Basal diameter (8.1 ± 1.6 mm) of the lateral roots corresponds to that of 3-go-Kanzo, which is the trading standard of Dongbei-Gancao used by the primary wholesale dealers at the producing districts.

GL-content of G. uralensis roots cultivated in the eastern Nei-Meng-gu (Fig. 4, Table I)

Because GL-contents of 1-year- ($0.98 \pm 0.24\%$, n=7) and 2-year-old roots ($1.03 \pm 0.28\%$, n=7) were on a low

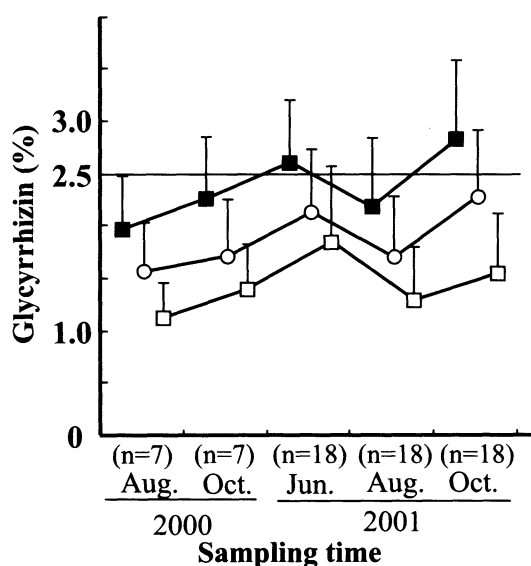


Fig. 4 GL-contents (%) of 3- and 4-year-old roots of *G. uralensis*. Each value represents the mean \pm S.D.
 □ : taproot, ■ : lateral roots, ○ : whole roots.
 GL-content of 4-year-old stolon harvested in October was $2.36 \pm 0.85\%$ (n=6), which might be used as material for out-of-grade of Gancao such as Mao-cao (Mo-so in Japanese).

level, those of 3-year- and 4-year-old roots are discussed in detail. As shown in Fig. 4, there were recognizable changes in GL-contents with cultivation time, thus 4-year-old plants in the flowering time (August) were poorer in GL-content than those in June. Although GL-contents of 4-year-old taproots harvested in October, 2001 ($1.52 \pm 0.57\%$) did not exceed 2.5%, those of 4-year-old lateral roots harvested in June ($2.58 \pm 0.62\%$) and October ($2.81 \pm 0.76\%$) in 2001 were in conformity with the JP XIV standard.

Therefore, 4-year-old lateral roots may be a candidate to complement insufficiency of *Glycyrrhizae Radix* caused by restriction of wild *Glycyrrhiza* plants collection in China. The 4-year-old lateral roots also conformed to the 4 quality control tests prescribed in the JP XIV (Table I). Although characteristics of *G. uralensis* cultivated in northwest China are reported,⁶⁾ this is the first report to examine GL-content of *G. uralensis* cultivated in northeast Nei-Meng-gu. However, GL-content of the present 4-year-old lateral roots is lower yet than the mean value ($5.16 \pm 1.00\%$) of Dongbei-Gancao collected from 1986 to 2000 in Japan.¹⁾ Further investigations are needed to increase total yields and GL-contents of the cultivated lateral roots by breeding and devices of cultivation conditions such as transplanting conditions and fertilizations.

GL-content of 5- and 6-year-old "taproots" cultivated in northwest China was less than 2.5%, however, that of 4-year-old "lateral roots" cultivated in the present

Table I. Characteristics of 4-year-old roots of *Glycyrrhiza uralensis* cultivated in the eastern Nei-Meng-gu in China (harvested in October, 2001)

	GL-content (%)	EtOH-ext. content (%)	Loss on drying (%)	Total ash (%)	Acid-insoluble ash (%)
Lateral roots	2.81 ± 0.76	29.6 ± 3.6	4.9 ± 0.4	4.3 ± 0.7	1.3 ± 0.5
Taproot	1.52 ± 0.57	24.4 ± 4.3	4.6 ± 0.9	3.7 ± 1.2	0.8 ± 1.0
Whole roots	2.26 ± 0.65	27.5 ± 3.4	4.8 ± 0.6	4.1 ± 0.7	1.1 ± 0.5
JP XIV standard	2.5 or more	25.0 or more	12.0 or less	7.0 or less	2.0 or less

Each value represents the mean \pm S.D. (n= 18)

Lateral roots: newly growing roots from tail of original taproot after transplanting
 Taproot :correspondence of transplanted seedling root

The cultivation field is located in Yuan-bao-shan-qu, eastern region of Nei-Meng-gu, inner Mongolia of China. On the periphery of the field, *G. uralensis* grows naturally.

Seeds were sown on May in 1998 and the seedling roots were cut to about 20-25 cm length and transplanted on May in 1999.

examination was more than 2.5%. That depends on the differences in climate, soil and cultivation conditions such as without or with transplanting. The influences of transplanting on the roots growth and GL-content are under investigation now.

GL-content of G. uralensis roots related to the flowering and number of leaflet (Fig. 5)

It is considered that growth conditions may be due to differences flowering in the same cultivation year. As shown in Fig. 5, GL-contents of 4-year-old lateral roots with flowers are higher than that of lateral roots without flowers. Since the 4-year-old plants with flowers were a small minority composing only 10% of the whole, GL-content in the flowering time (August) was poor as shown in Fig. 4. Further study is necessary to arrange flowering in approximately the same period by improvement of cultivation conditions such as fertilization.

Number of leaflet is also considered to be one of the index of growth conditions and genetic factors, however, there is no significant correlation between GL-contents of 4-year-old lateral roots harvested in October and number of leaflet: $2.29 \pm 0.35\%$ (for 7 leaflet plants, $n=6$), $3.05 \pm 0.74\%$ (for 9 leaflet, $n=6$) and $2.37 \pm 0.49\%$ (for 11 leaflet, $n=6$). Since correlation between phylogenetic type and chemical constituents of *Glycyrrhiza* plants are previously examined,⁷⁾ further breeding experiments are necessary to search *Glycyrrhiza* strains proper for the

medicinal use.

In summary, cultivation study in eastern Nei-Meng-gu of China was carried out in order to clarify how many years were necessary for *G. uralensis* to be cultivated to fit with the JP XIV standard. It was proved that GL-content of 4-year-old lateral roots, which newly grew from transplanted seedling taproot, exceeded the JP XIV standard (2.5% or more). The results revealed that cultivated *G. uralensis* might be a candidate of medicinal resources, however, the GL-content ($2.81 \pm 0.76\%$) of 4-year-old lateral roots was poor yet. Further examinations on improvement of cultivation conditions to obtain suitable length and thickness of lateral roots, high GL-contents and adequate ingredients-composition similar to wild *Glycyrrhiza* roots currently in use are in progress in the field in China.

Acknowledgement

Authors wish to thank Prof. P. Basnet (The School of Pharmaceutical Sciences, Pokhara University, Nepal) for his helpful discussion.

和文抄録

中国において野生甘草 (Gancao) の採集が砂漠化を誘発するために2000年に制限する通知が出され、栽培甘草の実用化が強く望まれるようになっている。本研究は、地球環境を保全し甘草資源を確保 (野生甘草の不足分を栽培甘草で補填) する観点から企画された。本論文の特徴は、*Glycyrrhiza uralensis* FISCH. を内蒙古自治区の東部 (元宝山区古山鎮西六家村：東北甘草の産地) において栽培した点にある。なお試験植物が *G. uralensis* であることは、3-4年目の夏に開花した花と果実および小葉の形態から確認した。

5月に播種し翌年の5月に苗を掘り上げ根を20-25cmに切りそろえた後、移植した。移植根から新たに生育した4年目の側根 (lateral roots) の glycyrrhizin (GL) 含量が、日本薬局方の基準値 (JP XIV: GLを2.5%以上含む) を越えることが明らかになった。中国の東北甘草の生産地域で栽培された *G. uralensis* の GL 含量を栽培年数と関連付けて検討したのは、本論文が初めてである。今回の実験によって、栽培甘草が薬用資源として利用できる可能性が高まった。しかしながら、4年目のGL含量 ($2.81 \pm 0.76\%$) は日本市場の東北甘草 (Dongbei-Gancao) の平均値 ($5.16 \pm 1.00\%$) と比べて満足できる

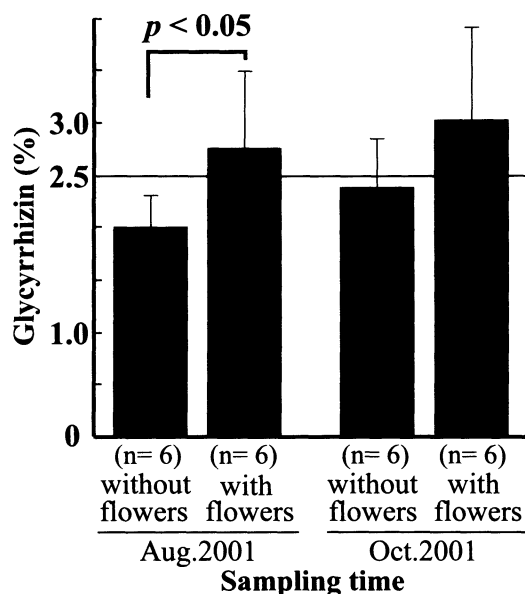


Fig. 5 GL-contents (%) of 4-year-old lateral roots of *G. uralensis* with or without flowers
Each value represents the mean \pm S.D.

値に達していない。優良種苗の選抜や栽培方法の改良など今後の検討が必要である。

この栽培実験と併行して中国の甘草生産地において栽培された *Glycyrrhiza* 根を調査したところ、JP XIV 基準に適合する物は得られなかった。なお、西北地域で蒐集した試料は移植せずに5-6年間栽培された主根 (tap-roots) であった。今回我々が実施した栽培実験において JP XIV の基準に適合したのは、移植後に主根から生育した側根 (lateral roots) であった。移植の有無や移植時期が側根の成長やGL含量に及ぼす影響に関しては、現在も内蒙古東部の畑において検討中である。

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