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ORIGINAL ARTICLE

Cultivation of Paeoniae Radix Rubra in the Wild-like Environment and the Evaluation of Quality for Transplanted Products

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ABSTRACT

Aim: The study aims to guide the actual cultivation of *Paeonia lactiflora* Pall. By establishing indoor and outdoor imitation wild environment, meanwhile, it used the quality inspection of cultivated production to judge the rationality of the scheme.

Method: The seeds and rhizomes of *Paeonia lactiflora* P. were collected in the autumn of 2014, and the seed germination rate was tested under indoor wild-like conditions. In the outdoor wild-like environment, the buds were transplanted, then the roots were harvested in 2016, 2017, and 2018, respectively. Quantitative determination of paeoniflorin was determined by HPLC. Furthermore, the data on trait determination and component content were compared and analyzed.

Results: In the specific indoor environment, the seeds were normally germinated, and their functions consisted of seed in wild regions. Moreover, the content of paeoniflorin in both two sources of *Paeonia lactiflora* P. roots, and the traits of cultivated products are increasing year by year.

Conclusion: The simulated wild environment established the method in this study, which is suitable for the artificial production of wild *Paeonia lactiflora* P.

Keywords: Cultivation, HPLC, Red peony seeds, Wild-like environment.

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INTRODUCTION

Paeonia lactiflora Pall is a perennial herb distributed in Russia, China, and other areas.[1] As one of the traditional Chinese medicines (TCM), red peony has an effect of clearing heat and cooling blood, dispersing phlegm and relieving pain,[2] which was applied in the treatment of warm venom hair spots, blood and heat spitting, red eyes and swelling, swollen sore sores, liver stagnation hypochondriac pain, menstrual dysmenorrhea embolism. The 2015 edition of the Chinese Pharmacopoeia stipulates that roots of Chinese herbaceous red peony are derived from the dried roots of *Paeonia lactiflora* Pall. or *Paeonia veitchii* Lynch.[3] Chinese herbaceous red peony is widely distributed in most parts of China. In the 1980s, Hebei province and Inner Mongolia Autonomous Region were the main producing areas, especially in Duolun County, being considered as genuine producing area. According to the resource survey, it is shown that the growing area for wild red peony is shrinking. It means that the wild resources of wild samples are gradually decreased. Qiqihar City is adjacent to Inner Mongolia, having a suitable climate and environment similar to Inner Mongolia. It has an imitation of wild environment cultivated and cultivated by *Paeonia lactiflora* P.

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There are shortcomings in the sowing and propagation of red peony under natural conditions, not only requiring

a long time within slow germination but also having a low germination rate and untidy emergence. It has a great impact on artificial cultivation and seed testing and brings great difficulties to seed germination, seeding, and flower production. Therefore, mastering the dormancy and germination characteristics of the red peony plants and implementing scientific sleep technology is the significance for improving cultivation efficiency, speeding up the cultivation of new varieties, and protecting and utilizing wild resources. It is obvious to improve cultivation and production efficiency, accelerating the cultivation of new varieties, and protecting and utilizing wild resources by mastering the characteristics of dormancy and germination and implementing reasonable sleep technology. Besides, in view of the long duration of seed sowing, the bud seeding method is the more common mode of production. This study is committed to expanding the planting species in the region and implementing the cultivation of Chinese herbal medicines. We artificially cultured wild root under the conditions of the simulated wild environment. The technology is applicable to stimulated wild-type cultivation of *Paeonia lactiflora* P. in Northeast China; meanwhile, the quality analysis was performed by necessary component analysis to provide the essential technical support for the production, raising production, sustainable development of *Paeonia lactiflora* P. in the stimulated wild environment.

IMITATION WILD LABORATORY (FIELD) SUMMARY

In winter, the temperature is a range of -10~-38 °C in the field. Therefore, the temperature range of indoor imitation wild room is controlled at -15~-50°C, and humidity is 40~60% RH. Outdoor imitation of wild environment locates in the medicinal botanical garden of Qiqihar medical university (longitude: 123.94378, latitude: 47.3767, altitude: 146 m), and planting area is nearly 6600 m^2. The soil is sandy loam, and the soil is loose and gentle. The experimental field has enough light. Before the planting wild samples, soybeans (*Glycine max* (Linn.) Merr.) is planted in this experimental field to fix the nitrogen element.

MATERIALS AND METHODS

Experimental Materials and Instrument

Instruments: Waters E2695 high-performance liquid chromatography (American Waters Technology Co., Ltd.), 752N UV-VIS spectrophotometer (Shanghai Yidian Holdings (Group) Co., Ltd., China)

Reagents and herbs: methanol (Merck Chemical Technology Co., Ltd.), potassium dihydrogen phosphate, chloroform, ethyl acetate, formic acid, deionized water. The methanol is the chromatographic grade, and other reagents are of analytical grade.

Experimental samples: The seeds and roots were collected in the wild side. The wild scorpion seeds were collected on the south side of a hillside in Zhalantun, Inner Mongolia, which was identified as *Paeonia lactiflora* P. by Associate Professor Jikai Sun of pharmacy college in Qiqihar medical university. The tested samples concluded wild picking samples, annual product, and biennial products as well as three-year-old cultivated products, namely, W, C1, C2, and C3. Reference substance: Paeoniflorin standard substance (China Food and Drug Administration Institute, the batch number is 110736-201842), namely, R. Besides, TLC samples concluded W, C3, and commercial product (C).

Statistics Analysis of Seed Hard Rate

Twenty seeds were randomly selected and immersed for 24 hours at room temperature to count hard seed. The test was repeated three times, and the average value was calculated.

Calculation formula: Hard solid rate = number of hard seeds/number of seeds tested

Screening of Seed Storage Conditions

The experiment was started in October of 2014, and ended in April of 2015 to study the optimum storage temperature and suitable time. The details were: the seeds were taken and immersed in a 0.5% potassium permanganate solution for 20 minutes and then rinsed. All seeds were divided into three groups, namely, -20°C group, -40°C group, and room temperature group. During the period of the experiment, we recorded the data every 7 days. The germination rate of the seed was measured in each group, and the germination rate of seeds after wintering by the above storage method was compared.

Analysis of Seed Germination Rate

The seeds were placed in a petri dish, which was placed into a filter paper as a germination bed, and water was added in an appropriate amount. The seed, filter paper, and petri dishes were sterilized with 0.1% mercuric mercury for 2 minutes, and cultured at a constant temperature of 25°C. The germination was investigated five days later. Each group repeated three times, and 20 capsules were in each group.

Germination rate = number of germination/number of seeds tested

Transplanting and Harvesting

Open shallow trench, and the depth was 5~7 cm. Wild root buds indoors were transplanted into the outdoor wild-type experimental field and cultured at a 30 cm distance. The buds

were upwards, and the buds were set with soil. Then, the decomposed cake fertilizer or organic fertilizer was applied to the ditch, and a small amount of soil was covered to keep a little pressure. We collected the root in the autumn of next year, the second year and the third year after the transplant. The diameter and fresh weight were simultaneously measured. Finally, dried roots were stored at -80°C.

Quality Inspections of Cultured Production

According to the Chinese Pharmacopoeia 2015, vol. 1, the indicator component paeoniflorin was determined. Sample preparation: we accurately weighed the appropriate amount of paeoniflorin reference substance, and added methanol to gat reference solution with 0.50 mg·ml^{-1}. Then we accurately weighed about 0.5g *Paeonia lactiflora* P. power originated from both the wild products and cultured products in different years. 25 mL methanol was precisely added in each sample to be treated by ultrasound after soaking for 4 hours. Then the weight loss was composed after cooling. Shake into uniform and pass 0.45μm microporous membrane by taking the filtrate.

Detection Conditions: Chromatographic column was Agilent ZORBAX Eclipse XDB-C_{18} column (250 × 9.4 mm, 5 μm); mobile phase was 0.05 mol·l^{-1} methanol-potassium dihydrogen phosphate (50:50). The flow rate was 1.0 mL·min^{-1}, and the detection wavelength was 230 nm with 10 μm injection amounts.

Data Processing

Experimental data were processed by SPSS 20.0 and 2016 Microsoft Excel. The experimental results were expressed as mean ± SD.

RESULTS

Seed Hard Rates

There were no hard seeds by the hard-solid rate test, which ensured the consistency of the experimental seed.

The Germination Rate of Seeds in the Wild-Like Environment

The best store condition of seed was at -20°C for 2 months, and -40°C for 1-month and the rooting rate was 38% higher at -20°C for 2 months than the control group. The average length of root was 8.03 cm after treatment for one month at –40°C, which was 3.07 cm longer than the control group. The results were shown in Table 1 and Figure 1A is a photograph of seed germination.

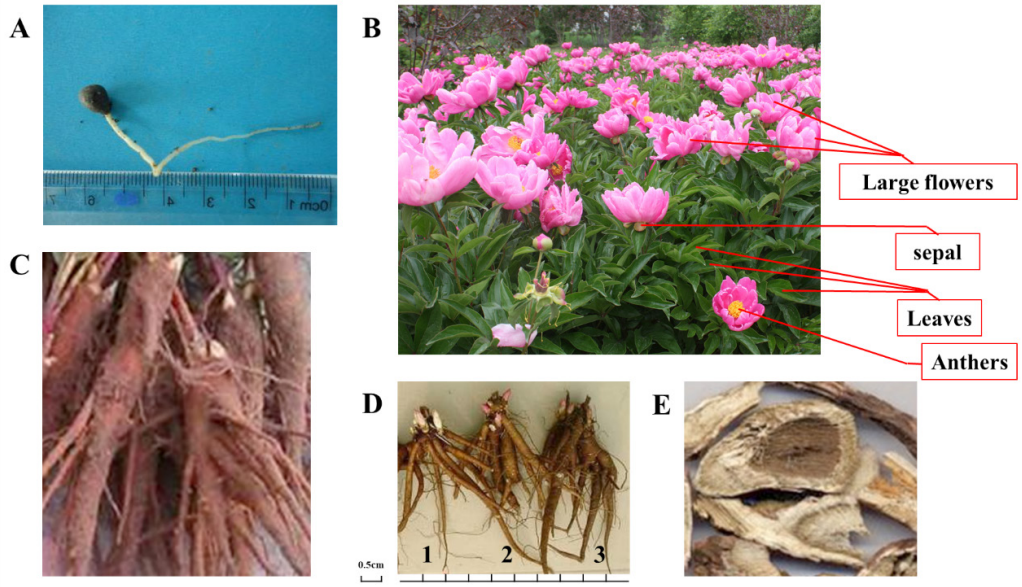

Figure 1: The growth of *Paeonia lactiflora* P. in the wild environment. A Photograph of one seed germination in 60th d at -40°C. B Plant of the cultivated product in a wild-like environment. C. The root of the excavation is in the color of reddish-brown. D. The roots are cultured in the 1 to 3 years. E Cross-section of dried root of cultivated products with "skin sputum".

Table 1: The growing state of seeds in the wild-like environment during experience period (n = 20)

		-20°C				*-40°C*			
	con	*30th d*	*60th d*	*90th d*	*120th d*	*30th d*	*60th d*	*90th d*	*120th d*
Germination rate (%)	50	70	85	88	73	89	86	77	50
Average length (cm)	4.10	5.71	5.92	4.24	7.31	8.03	6.82	6.67	2.50

Characteristics of Outdoor Cultivation Products

It's seen that the heights were not much different in the annual, two-year, and three-year-old cultivated plants, nearly in the range of 70~80 cm. The leaves were alternate, the leaves were nearly leathery, having stalks about 6 cm long and the leaflets are oblong. Leaflets were green and glabrous, and the shape was oblong and elliptic within the acuminate apex and cuneate base. Large flowers were terminal and glabrous within long stalk, and it also had leaf blade sate and 3~5 sepals that was obovate ovate. Petals were pink in the color of obovate or obovate-elliptic. Anthers were yellow. Commonly, carpels were 2~5 and glabrous. Stigma is brownish-purple. (Figure 1B) The root was spindle-shaped or cylindrical hypertrophy in the reddish-brown color, and the folded section was pink white. (Figure 1C) The diameters of the fresh roots in annual, biennial, and three-year-old were increased year by year. (Figure 1D) The dried root of cultivated products had the characteristics of "skin sputum." (Figure 1E).

Determination of the Indicator Components

The HPLC baseline was stable. The chromatogram showed that all wild samples, annual/biennial/three-year-old cultivation products contained paeoniflorin. Furthermore, the contents were no significant differences in each group, and the three-year-old cultivation products had the highest content. The results indicated that the main chemical composition of the cultured products consisted of wild ones (Figure 2, Table 2). At the end Table S1 showed detailed information of precision test and stability test. Moreover, the accumulation of paeoniflorin was also increased with growth periods. Furthermore, TLC was also performed to briefly compared the quantity of ingredients among R, W, C3, and Y (Figure 3). The diameter of spots was higher in the cultivated products than in the commercial group, indicating that the wild environment of the area was preliminary. The cultivated products should be greater than the commercial products.

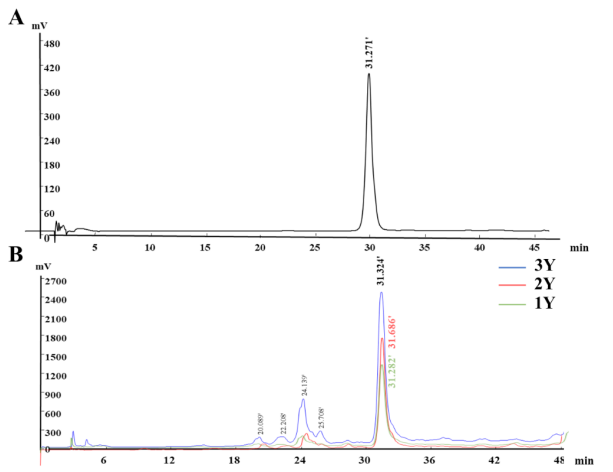

Figure 2: HPLC spectrum of reference and cultivated production under the same condition. A. HPLC chromatogram of paeoniflorin; B. HPLC chromatogram of cultivated production in the 1 to 3 years (1Y to 3Y). From A and B, t_R = 31' is retention time of indicator components.

DISCUSSION

Impact of the Ecological Environment on the Quality of *Paeonia lactiflora* P.

Paeonia lactiflora P. is one of species in red peony genus. In China, red peony genus has total of eight species and

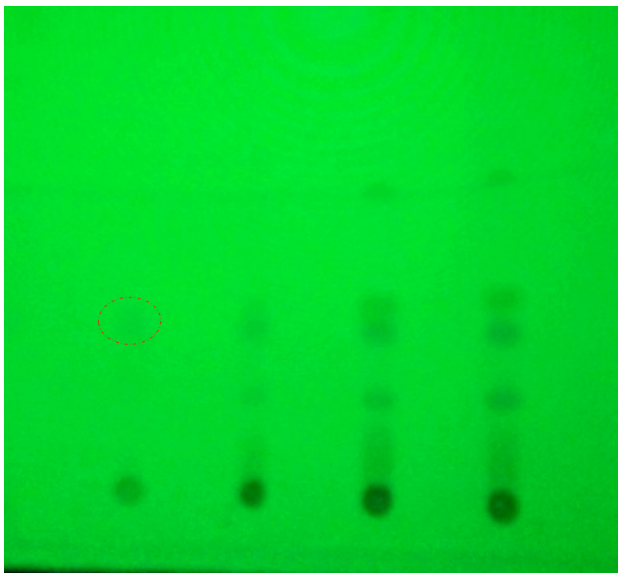

Figure 3: TLC chromatogram for radix of *Paeonia lactiflora* P. from different sources. The samples were R, W, C3 and Y from left to right in the figure, and red circle signed the paeoniflorin.

Table 2: The peak area of samples in HPLC (n=2)

	R	*C1*	*C2*	*C3*
Sample 1	6915507	13356195	17769934	20861843
Sample 2	6932131	13439674	21103120	20866331
Average	6923819	13397934.5	19436527	20864087
Content (%)	11.61	4.41	6.55	7.02
RSD (%)	0.8	0.29	0.16	0.80

six varieties widely distributing in Northeast China, North China, Northwest China, *etc*. The climate contains the cold temperate zone, the middle temperate zone, the warm temperate zone, the northern subtropical zone, the mid-subtropical zone, and the plateau climate zone. Thus, it owns different growth environments, including soil quality, soil pH, and water conditions, which affect the plant's growth, especially the accumulation of secondary metabolites. It is well known that different hormones and signaling substances interact with each other in the optimal pathway to accumulate secondary metabolites while herbs accept stimulation from the environment. The meaning is possible for secondary metabolites. The synthesis and accumulation of secondary metabolites is the result of medicinal plants being shaped by the living environment in which they are located in.[4-6] The growth environment makes a huge impact on the accumulation of secondary metabolites, [4-7] and is also influenced on quality of medical herbs. Therefore, it is necessary to investigate the imitation of the wild environment on the secondary metabolites when exploring the cultivation and translation of the medicinal materials. It is critical to rationally construct the imitation ecological environment to ensure the quality of artificial products. Meanwhile, the cumulative amounts of secondary metabolites can reflect the similarity between the wild environment and the cultivation environment. In this study, we chose the wild-type test plots adjacent to natural origin. The climate, sunshine, soil, and other ecological environments were very similar to the wide. In theory, there was no difference in the quality of the red peony produced by the two places, and the experiment determined the paeoniflorin to verify the quality of cultured products of *Paeonia lactiflora* P.

Technical Progress of *Paeonia lactiflora* P. Cultivation

Resources of red peony have fallen sharply, and red peony is suited in the northeastern provinces and northeastern Inner Mongolia of China. In recent years, many researchers and growers have carried out many useful explorations. At present, artificial production methods of red peony radix mainly include seed sowing and germination. However, red peony seed has a special characteristic of double-dormant germination, which makes the red peony seed germination take a long time. The unfavorable result is that germination is irregular and the germination rate is low, which causes the dilemma of inspection, cultivation, sowing seedling, and flower production. Therefore, double dormancy must be re-edited, especially after breaking the hypocotyl dormancy and breaking the epicotyl dormancy, then the red peony seed can germinate. Otherwise, there is just a phenomenon of rooting without germination. The technology for breaking the dormancy mainly includes machinery breaking technology, chemical reagent treatment, and hormone treatment technology, *etc*. In this study, we used low-temperature treatment technology to play a qualitative role in relieving dormancy.[8-11] It not only shortens the germination time of red peony seed but also guarantees the normal sowing in the spring of the following year. In the course of operation, care should be taken to ensure that the seeds are evenly cooled, and the piles of seeds should not be too large. If the unevenness of the cold will break the dormancy, the seedlings will not be ready after planting.[12]

Paeonia lactiflora P. can be used in medical and ornamental fields, and the market demand is growing. In recent years, due to the increase in demand, the resources of red peony have been in short. In Jilin, the stimulated wild red peony has been made in the Inner Mongolia, Heilongjiang, Anhui and other places. The usual fields are land for growing field crops or the land under the fruit trees.[13] Due to the long growth cycle of *Paeonia lactiflora* P., and wild products are still the main source. Large-scale production would be an effective way to relieve the current market supply and demand, corresponding with the lack of wild resources. However, the high investment costs limit the development of manual production. At present, the area of artificially planted red peony is currently small, which is the main reason for the rising market price. This study aims to establish a wild-type seed germination environment of *Paeonia lactiflora* P. to shorten the seed germination cycle and imitate the wild cultivation techniques to maintain the consistency of the habitat. The results showed that the seeds can be germinated under the experimental conditions. The transplanted products and the plants are no different from wild products in the outdoor experimental conditions.

CONCLUSION

In the indoor/outside wild-type environment, the study optimized the conditions suitable for the germination of *Paeonia lactiflora* P. seed to shorten the germination cycle and investigate the transplanting roots of *Paeonia lactiflora* P. in different years. The detection of index components to evaluate the quality of products under the stimulated wild-environment. The results showed the feasibility of the method. The simulated wild environment constructed in this experiment was suitable for planting and transplanting red peony seed, and can be used as a guide for agricultural technology.

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CONTRIBUTION OF AUTHORS

Zhang Honglian: organize data and write articles; Zhang Meijuan: guide seed germination test; Li Hongling: do the HPLC test; Dong Wei: proceed the data of HPLC; Sun Jika: design the experience; Liu Yuliu: access related literature and typesetting.

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APPENDIX

Table S1: Precision test and stability test for HPLC

Index	*Precision*	*Time (h)*	*Stability*
1	6813631	0	139844974
2	6915507	2	139120973
3	6932131	4	139000865
4	6946132	8	140098770
5	6962139	24	130864356
6	6913908	48	139874322
RSD (%)	0.8		1.5