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Impact of phosphorus and potassium fertilizers on growth and anthraquinone content in *Rheum tanguticum* Maxim. ex Balf

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ABSTRACT

The dried root of *Rheum tanguticum* plays an important role in formulations and prescriptions in traditional Chinese medicine and Kampo medicine. Due to over-exploitation, *R. tanguticum* resources have decreased sharply in recent years. The main objective of our investigation (a 3-year field experiment) was to explore the effect of different levels of phosphorus (superphosphate) and potassium (potassium sulfate) fertilizer on the biomass (root fresh weight, root increment, and root dry weight), yield, dry matter content, and anthraquinone content of this plant at different harvesting stages (green stage, growth stage, and wilting stage) under alpine conditions. The root fresh weight and root dry weight increased significantly at the wilting stage following treatment with 90 kg P_2O_5/ha (100% and 59%, respectively) in 2016 and 75 kg K_2O/ha (43% and 41%, respectively) in 2015 compared to the control. The yield of root dry weight obtained from three-year-old *R. tanguticum* plants was 9200 kg/ha when 90 kg P_2O_5/ha of phosphorus fertilizer was applied, and 10,400 kg/ha when 75 kg K_2O/ha of potassium fertilizer was applied. This yield reached a maximum at the wilting stage. The anthraquinone content of two-year-old *R. tanguticum* plants had already reached the standard level of the *Chinese Pharmacopoeia*; however, three-year-old plants had double the anthraquinone content of two-year-old plants. Phosphorus and potassium fertilizers had no obvious influence on the anthraquinone content of *R. tanguticum* at the same harvesting stage.

1. Introduction

Rhubarb, one of the ancient and best-known Chinese herbal medicines, is officially listed in the *Chinese Pharmacopoeia* as containing three species, *R. palmatum* L., *R. tanguticum* Maxim. ex Balf. (*R. tanguticum*), and *R. officinale* Bail (*R. officinale*). It has been used for thousands of years in China (Li et al., 2014). Rhubarb is also officially listed in the *Japanese and European Pharmacopoeia*. *R. tanguticum* is one of 39 species of *Rheum* distributed in China, and is typically endemic in the eastern part of the Qinghai-Tibet Plateau. Moreover, *R. tanguticum* is of better medicinal quality than the other two species. It grows on alpine rocks and slopes at an altitude of 2500–4400 m in the temperate and subtropical regions of the world (Wang, 2009).

R. tanguticum has long been used as a purgative agent, an anti-blood stagnation and anti-inflammatory agent, and a cure for gastric and renal disorders (Wang et al., 2008; Zhang and Liu, 2004). Phytochemical investigation has revealed that five anthraquinones—aloe-emodin, rhein, emodin, chrysophanol and physcion—are the major active compounds in this plant. These five compounds serve as the quality control standards for the *Chinese Pharmacopoeia*. In addition to its pharmacological values, the young stems and petioles of this species are a nourishing food.

In recent years, wild resources of *R. tanguticum* have decreased rapidly year on year, and it has become an endangered species because of its very limited distribution, poor living habitat, over-harvesting, and increased market demand (Li, 2014). The demand for *R. tanguticum* continues to increase. Moreover, *R. tanguticum* is poorly known in Western countries even though it has been continuously used in China for thousands of years (Wang, 2009). Based on the analysis above, it is very desirable to establish standardized fertilizer practices to improve the yield and quality of *R. tanguticum* for its sustainable use as an effective medicine.

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Fertilizer can increase the biomass yield and effective constituent content of traditional Chinese medicine. For example, medium levels of nitrogen and phosphorus fertilizer significantly increased the biomass and saikosaponin A levels of *Bupleurum chinense* roots (Zhu et al., 2009). Phosphorus fertilizer has an effect on plant growth indexes and essential oil quality (Kapoor et al., 2004). Inorganic fertilizer causes a large increase in growth, chlorophyll content, and dry grain yield of maize and sorghum (Amujoyegbe et al., 2007). Furthermore, increased K_2O levels resulted in higher grain and oil yield per hectare in the first year in crambe (Jid et al., 2013). It was also reported that *R. tanguticum* requires highly fertilized soils to attain good productivity (Zhang, 2012). However, there have been few investigations on the effects of fertilizer on *R. tanguticum*. Therefore, the objectives of this study were to explore the effect of different levels of phosphorus and potassium on *R. tanguticum* growth, and to optimize a rapid and sensitive reversed-phase high-performance liquid chromatography (HPLC) method to quantify the anthraquinone content (aloe-emodin, rhein, emodin, chrysophanol and physcion) in *R. tanguticum*.

2. Materials and methods

2.1. Location and climate conditions

A field experiment was conducted during 2014, 2015 and 2016 at the Donggou Township of Huzhu County, Qinghai Province, China (36°50′15″N and 101°57′06″E), at an average altitude of 2500 m. The chemical properties of the soil used in this study are shown in Table 1. The precipitation and temperature data were provided by the Weather Bureau of Huzhu County, Qinghai, China. This area has a typical plateau continental climate. The mean precipitation for 2013–2016 ranged from 35.6 to 42.2 mm/month. The mean maximum and minimum temperatures were between −0.7 to 31 °C and −23 to 16 °C, respectively.

2.2. Experimental design

2.2.1. Treatment

The experiment was arranged in a completely randomized block design with eight treatments and three replications. Phosphorus was applied as super phosphate (12.0% P_2O_5) at a rate of 0 (P0), 22.5 (P1), 45 (P2), or 90 (P3) kg P_2O_5/ha; and the potassium used was in the form of potassium sulfate (50.0% K_2O) at rate of 0 (K0), 37.5 (K1), 75 (K2), or 112.5 (K3) kg K_2O/ha. The plants were fertilized twice during the growth stage (early July and end of August).

2.2.2. Sowing and harvest

R. tanguticum seeds were identified by Professor Guoying Zhou and collected from Huzhu County, Qinghai, China. The voucher specimen was deposited in the herbarium of the Northwest Institute of Plateau Biology, Xining, Qinghai, China. Seeds were sown in a 1 m × 1 m nursery bed in May 2013. One-year-old seedlings were weighed before being transplanted into the field plots in May 2014. Each plot size was on average 120 m^2, and plant spacing × row spacing was set at 50 cm × 50 cm. Plants were harvested in 2015 and 2016 at the green stage (early May, when showing regrowth of the stems and leaves), the growth stage (mid-July, with leaves and stems growing rapidly, and showing signs of bolting), or the wilting stage (early October, above-ground parts withered). Ten plants from every plot were collected in a randomized block design for each replicate. Shoots and roots were separated. The fresh weight of each root was measured after each collection, the fresh roots were dried at 45 °C to a constant weight, and then reweighed. We analyzed root fresh weight (FW, g/plant), root increment (root fresh weight – seedling fresh weight before transplanting into plot, g/plant), and root dry weight (DW, g/plant).

Table 1
Chemical properties of the experimental soil.

Test item	Value	Test item	Value
pH	7.12	Salt content(g/kg)	0.99
Total nitrogen (g/kg)	1.77	Available nitrogen(mg/kg)	139.83
Total phosphorus (g/kg)	0.719	Available phosphorus(mg/kg)	28.67
Total potassium (g/kg)	21.67	Available potassium(mg/kg)	48.3
Total carbon (g/kg)	27.02	Electrical conductivity(mS/cm)	9.88×10^{-3}
Organic matter(g/kg)	37.7	Organic carbon (g/kg)	21.87

2.3. Materials and reagents

The reference standards for aloe-emodin, rhein, emodin, chrysophanol, and physcion were purchased from the National Institutes for Food and Drug Control (Beijing, China). Ultrapure water was obtained from a Millipore Milli-Q system (Bedford, MA, USA). HPLC chromatographic grade methanol was purchased from the Yu Wang Group (Shandong, China). Other chemicals were analytical grade. Chromatographic analysis was performed using the Agilent 1260 system, with a G1311A quat pump, G1315D DAD detector, G1329A autosampler, and Agilent HPLC software (Agilent, USA). The chromatography columns (4.6 × 250 mm, 5 μm, 100 Å) were purchased from Acchrom Technologies Co., Ltd., China.

2.4. Sample solution and standard solution preparation for HPLC quantitation

The sample solution was prepared according to the *Chinese Pharmacopoeia*. The dried materials were pulverized in an electric grinder, and the resulting powder sieved through 177 μm sieves. The powdered herbal sample (0.150 g) was heated for reflux extraction with methanol (25 mL) for 1 h, cooled at room temperature, and then filtered. The filtrate (10 mL) was transferred to a flask for solvent evaporation, and then treated by ultrasonic extraction using HCl/H_2O (10 mL, 10:35, v/v) for two minutes, refluxed extraction with trichloromethane (10 mL) for 1 h followed by cooling at room temperature, and extraction with trichloromethane (10 mL × 3). The extracting solution was merged for rotatory evaporation until a yellow residue formed, which was dissolved in methanol (10 mL) to generate the sample solution. The sample solution was filtered through a 0.45 μm organic membrane prior to injection into the HPLC system.

A separate standard solution containing 172 μg/mL aloe-emodin, 112 μg/mL rhein, 172 μg/mL emodin, 160 μg/mL chrysophanol, and 156 μg/mL physcion was prepared by dissolving the chemicals in methanol. Aloe-emodin, emodin, chrysophanol, and physcion (0.2 mL each) and rhein (0.3 mL) were transferred into 10 mL volumetric flasks. The HPLC system was calibrated for total anthraquinones by using the standards at injection volumes of 2, 5, 10, 15, 20, 25, 30, 35, 40, and 50 μL.

2.5. Chromatographic conditions

Mobile phase A was 0.2% v/v formic acid in ultrapure water and mobile phase B was methyl alcohol. The flow rate was 0.8 mL/min. The column temperature was 25 °C. The linear gradient elution was as follows: 0–30 min, 55–75% B; 30–60 min, 75–90% B. Chromatograms were recorded at 280 nm.

2.6. Statistical analysis

The data were expressed as mean ± standard deviation (SD). The statistical analysis was performed using OriginPro 8.5. Analysis of variance was carried out using IBM SPSS Statistics 20.0. Statistical significance was set at $P \leq 0.05$ and determined using Duncan's multiple range test.