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Research Paper

Effects of manganese on accumulation of Glycyrrhizic acid based on material ingredients distribution of *Glycyrrhiza uralensis*

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ABSTRACT

Wild and cultivated Licorice (*Glycyrrhiza uralensis* FISCH.) are the main source of licorice. In recent years, wild *Glycyrrhiza* plants have been seriously damaged and this has reduced the population. The cultivation of Licorice has been the best way to solve the problem of its shortage. The contents of glycyrrhizic acid greatly differs in licorices cultivated at different region of China. However, the contents of glycyrrhizic acid in cultivated licorice in china is lower than that of wild licorice. To produce the high quality cultivated licorices, researchers studied the factors affecting the glycyrrhizic acid produced in licorice. It was found that manganese (Mn) application improved the medicinal material yieldand quality especially the contents of the glycyrrhizic acid. In this paper, we investigated the effects of Mn on the accumulation of glycyrrhizic acid in medicinal materials based on the material ingredients distribution and also explored the mechanism. The influence of Mn on the relative contents of glycyrrhizic acid were significant except for day 30 while the influence is significant to the absolute contents all the time. The relative contents of glycyrrhizic acid increased while the contents of crude protein, crude fiber and total material ingredients decreased. The absolute contents of glycyrrhizic acid rose when crude protein contents increased. This implies that the contents of glycyrrhizic acid are in a close relationship with material ingredients. Mn application of appropriate concentration could stimulate the formation of primary metabolites, and then promote the secondary metabolism of *G. uralensis* which could lead to the formation and accumulation of glycyrrhizic acid in *G. uralensis*.

1. Introduction

Licorice (*Glycyrrhiza uralensis* FISCH.) belongs to perennials of the genus *Glycyrrhiza* from the family of Fabaceae, which is most widely used medicinal plants in traditional Chinese medicine (Pan et al., 2006; Dong et al., 2014). Licorice is also an important ingredient in a variety of Chinese herbal preparations, which is widely used to treat respiratory, gastrointestinal, and cardiovascular diseases (Wang et al., 2012). Glycyrrhizic acid extracted from licorice root and rhizomes (stolons) are the main officinal active components of licorice medicine quality, and its content impacts clinical efficacy (Yang et al., 2007; Tang et al., 2008). In recent years, wild *Glycyrrhiza* plants have been seriously damaged and this has reduced the population. Licorice cultivation has been the best way to resolve the shortage of licorice (Wang et al., 2012). Therefore, it is inevitable that licorice cultivated species will soon replace wild (Lu et al., 2008; Hou et al., 2010). However the content of glycyrrhizic acid is greatly different among the cultivated licorice of different region but are lower than wild licorice in China. Therefore, as the most consumed herbs in traditional Chinese medicine and an important sourceof pharmaceutic preparations in the modern medicine (Wang et al., 2006; Zhou et al., 2010), how to increase the content of glycyrrhizic acid and produce the high quality cultivated licorice is an urgent issue in the research industry and production fields.

Manganese (Mn) is an essential nutritional element necessary for activation of a wide range of enzymes and an indispensable constituent

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of the catalytic center of enzymes such as Mn-superoxide dismutase and the water splitting complex (Ducic et al., 2006). In addition, Mn application improves accumulation of active constituents in medicinal plants (Chen and Wu, 2007). The trace element of Mn has significant effect on the contents of aloin and aloe-emodin in *Aloe barbadensis*. When the concentration of Mn in the nutrient solution was 1.0×10^{-5} mol/L, the contents of aloin and aloe-emodin were high (Li et al., 2010). Mn could promote polysaccharide contents in continuously cropped *Chuanminshen violaceum* (Zeng et al., 2007). Mn could not enhance or affect indistinctively the relative contents of total flavones and l-borneol, but increased significantly the absolute contents of total flavones and l-borneol. The absolute contents of l-borneol under 4.0 g/L Mn treatment were significantly higher than those of other treatments (Wang et al., 2014). Thus, Mn is an important trace element for medicinal plants.

It's well known that the contents of glycyrrhizic acid in *G. uralensis* are affected by many factors, such as genetic differences (Kojoma et al., 2011), environmental conditions (Yu et al., 2015), fertilization (Wang et al., 2010) etc. Fertilization is one of the most effective methods to promote the growth of plants and the contents of active components. Wang et al. (2012), Ma and Wang (2015) had verified that Mn application improved the growth indicators, physiological indicators, medicinal material yield, and quality, especially the contents of the glycyrrhizin acid of *G. uralensis* (Wang et al., 2010; Ma and Wang, 2015). In licorice that the medicinal active ingredients (i.e., glycyrrhizic acid and liquiritin) that are considered as the secondary metabolites are only a few percentage, however, that the percentage of these material ingredients (e.g., total sugar, cude fiber, crude protein, crude fat, ash etc.) that are considered as the primary metabolites are higher. Therefore, these material ingredients constitute the main part of licorice. Usually the contents of medicinal active components refer to the percent of the quality of medicinal active components that are accounting for the total quality of crude drugs. In other words, the contents mean to the percent that the quality of medicinal active components are accounting for the quality of all material ingredients. Therefore, the quality of change of any material ingredient will affect the relative value.

The objective of the study was to determine the effect of Mn on accumulation of glycyrrhizin acid and material ingredients in *G. uralensis*, and also to discuss how the mechanism of material ingredients distribution affect the contents of glycyrrhizin acid. Results from this study could contribute to the analyses of factors influencing the quality of licorice, and provide some reference for producing a high quality cultivated *G. uralensis*.

2. Materials and methods

2.1. Plant materials

Seedlings of one-year-old *Glycyrrhiza uralensis* FISCH, identified as *G. uralensis* by professor Wenquan Wang who works at Beijing University of Chinese medicinewere collected from the same population in Wenniuteqi, Inner Mongolia Autonomous Region, China.

2.2. Experimental design

The roots of *G. uralensis* were grown in plastic pots (35 cm in diameter × 35 cm high) filled with vermiculite, perlite and soil (V/V/V = 5/1/1). The pots were embedded into soil in Beijing University of Chinese Medicine. Each pot contained twenty roots. The Hoagland nutrition solution was sprayed into the pot every week. The composition of the complete Hoagland nutrition (normal) solution was 506 mg/L KNO_3, 136 mg/L KH_2PO_4, 1180 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 493 mg/L $MgSO_4 \cdot 7H_20$, 1.81 mg/L $MnSO_4 \cdot H_2O$, 2.86 mg/L H_3BO_3 , 0.08 mg/L $CuSO_4 \cdot 5H_2O$, 0.22 mg/L $ZnSO_4 \cdot 7H_2O$, 0.52 mg/L $Na_2MoO_4 \cdot 2H_2O$, 2.5 mg/L Fe-EDTA. The pH of nutrient solutions was set at 6.5–7.0 (Zhang, 1990). The roots were first cultivated in the above-mentioned complete nutrient solution and then roots treated with incomplete Hoagland nutrient solution of five different Mn concentrations (0, 1.81, 18.1, 36.2 and 54.3 mg/L); the 1.81 mg/L was the normal concentration of Mn in the complete Hoagland nutrition solution. There were four replications for each treatment arranged in a completely randomized block design. Samples were analyzed on days 30, 60, 90, and 120 after treatment with different concentrations of Mn in the order listed above.

2.3. Indicator determination

The total sugar content was determined by Li method (2013) using phenol-sulfuric acid colorimetry. The crude fat content was determined according to Peng et al. method (2013) using residual method. The crude protein content was determined according to the method of Zhang et al. (2013) using Kjeldahl method. The crude fiber content was determined according to the method of Zhang et al. (2013) using acid-base washing method. The ash content was determined according to the method of "Chinese Pharmacopoeia" (2015) using high temperature burning method. The glycyrrhizic acid content was determined by HPLC method described in "Chinese Pharmacopoeia" (2015) using.

To describe the function of Mn in the accumulation of glycyrrhizin acid, the concepts of relative and absolute content were introduced in the study. Relative content of glycyrrhizin acid was referred to as the percentage of 100 units of quality licorice containing units of quality glycyrrhizin acid, i.e. usual content of glycyrrhizin acid (unit: %). Absolute content was referred to individual plant quality in licorice containing glycyrrhizin acid, i.e. individual plant production of glycyrrhizin acid (unit: mg/plant).

2.4. HPLC analysis

The dried roots of *G. uralensis* were powdered with pulverizer and filtered through 0.28 mm (60 meshes) sifter. The glycyrrhizic acid content was quantitatively analyzed by HPLC according to the following procedures. Dried samples (100 mg) were extracted with 10 mL 70% ethanol for 30 min under ultrasonication (250 W, 40 KHz, 40 °C). The solution was cooled to room temperature, adjusted with 70% ethanol to original volume, and filtered through analytical filter paper. The solution was filtered through a millipore filter (0.45 μm) before being injected into the HPLC system (Waters2489, USA), comprising of 2489 UV/Visible detector, 2707 autosampler, 1525 binary pump and Breeze 2 data processing system and a reverse-phase C18 column (C18, 4.6 × 250 mm, 5 μm particle size, Waters), with a column temperature of 25 °C, injection volume of 10 μL and detection wavelength of 237 nm for glycyrrhizic acid. Two mobile phases: solvent A (acetonitrile) and solvent B (0.05% H_3PO_4) and gradient profile: Time 0 (19% A), 8 min (19% A), 35 min (50% A), 36 min (100% A), 40 min (19% A), and the flow rate of 1.0 mL/min was used. The concentration of glycyrrhizic acid in the licorice was calculated by use of standard curves after triplicate analysis. The glycyrrhizic acid standard was from Chendu Must Bio-technology Co., Ltd. in Sichuan of China, with a purity of greater than 98% (Fig. 1).

2.5. Statistic alanalysis

Statistical analysis (one-way ANOVA, correlation) were performed using SPSS software (version 16.0, SPSS, Chicago, IL, USA); calculated statistical significance was considered at $p < 0.05$.

3. Results

3.1. Variation in relative and absolute contents of glycyrrhizic acid in G. uralensis

As indicated in Fig. 2, the relative contents of glycyrrhizic acid increased first and then decreased with increasing Mn treatment