

Breeding of new *Rehmannia glutinosa* variety

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Abstract

Huaidi 81, a new variety of *Rehmannia glutinosa* with excellent comprehensive characters, was screened by space mutation of hybrid seeds of 85-5 and Beijing No.1. The fresh weight, index composition, resistance, chlorophyll, anthocyanin and photosynthetic characteristics of Huaidi 81 and the main cultivars were determined. The results showed that: the per plant fresh weight of *Rehmannia* ranked in the order as follows: Huaidi 81 > 85-5 > Golden Nine > Huaifeng > Qinhuai > Beijing No.3, there was extremely significant difference between Huaidi 81 and others. The catalpol content ranked in the following order: Beijing No.3 (1.601%) > Qinhuai (1.588%) > Huaidi 81 (1.314%) > Golden Nine > 85-5 (1.073%) > Huaifeng (0.924%). There was no significant difference between Huaidi 81 and Golden Nine, but extremely significant difference between was found in Huaidi 81 and other varieties; The acteoside content ranked in the following order: Huaidi 81 (0.096%) > Qin Huai (0.069%) > 85-5 (0.047%) > Beijing No.3 (0.035%) > Huaifeng (0.023%) > Golden Nine (0.022%). There was significant difference between Huaidi 81 and other varieties. Huaidi 81 showed high resistance to *Septoris digitalis* Pass and middle resistance against leaf ring rot, which indicated that Huaidi 81 had good resistance to leaf diseases. Huaidi 81 with highest chlorophyll content and moderate anthocyanin content showed the highest photosynthetic rate. All these results indicated that the new variety Huaidi 81 with best comprehensive properties was suitable for popularizing as a new *Rehmannia glutinosa* variety.

Key words

Huaidi 81, Hybridization breeding, Index components, *Rehmannia glutinosa*, Photosynthetic rate, Space breeding

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Introduction

Rehmannia glutinosa Libosch with the longest cultivation history in (Zeng *et al.*, 2004) Henan "ancient Huaqing Fu" of the Geo-authentic areas. *Rehmannia* is one of the four Famous Huai Medicine (Wen *et al.*, 2002). According to different processing methods, *Rehmannia* can be divided into fresh rehmanniae, radix rehmanniae and prepared rehmannia root, they all have nourishing yin effect, but there are some differences (Li *et al.*, 2001). The fresh

Rehmannia is sweet and cold with cooling blood and hemostatic effect; the radix *Rehmannia* is sweet and cold with nourishing yin and producing saliva effect for hot blood, crimson tongue and thirst; and the prepared rehmannia root is sweet and warm, with nourishing the liver and kidney function, it is used for vertigo, tinnitus, osteopyrexia and hectic fever (Pharmacopoeia Committee of the Ministry of health of the people's Republic of China 2010). The yield and quality of original varieties have degenerated as a result of long-term asexual reproduction. Thus, in the spring of 2006,

breeding was carried out with hybrid seeds through natural hybridization by 85-5 as female parent and Beijing No.1 as male parent with good comprehensive resistance. The seeds were sent into space for mutagenesis with breeding satellite "Practice Eight" on September 9, 2006, through 7 years' breeding and propagation after returning to the ground, new variety Huaidi 81 with excellent characters was generated. Therefore, the aim of the present study is to compare the yield, quality, resistance, the chlorophyll, anthocyanin and photosynthetic rate of Huaidi 81 and to provide the main cultivars the theoretical and technical support for the cultivation and promotion of Huaidi 81.

Materials and Methods

Plant materials : In April 2013, six *Rehmannia* varieties Huaidi 81, Qinhui, Golden Nine, 85-5, Huai feng and Beijing No.3 were planted at three different spots in Wuzhi, in Xiazhuang Village, Bei Guo, Guo Village, Chengguan, Zhong Situ Village, Da Hongqiao, respectively. In every field trials, random test method was used for grouping six varieties. The varieties were managed according to the conventional field and the resistance identification was conducted in Xia Zhuang Village, Bei Guo Town in mid-September. Six varieties were harvested on November 23, 2013 in Xia Zhuang Village, Bei Guo. The weight of the plant and yield were tested, six varieties were harvested and the yield were tested on December 20 in Guo Village, Chengguan, in Zhong Situ Village, Da Hongqiao the six varieties were harvested and the yield were tested on November 25. Fresh *Rehmannia* were dried and crushed for determination of index components.

The autumn sprouts of six varieties were bred in Wen County in July 2014, then the six varieties were planted in Zhangsi Village, Wen Country on April 20, 2015 and managed according to the conventional field. The photosynthetic rate was tested on July 29, 2015. *Rehmannia* leaves were chosen randomly for chlorophyll and anthocyanin content.

Main instrument : Agilent 1200 series HPLC System, equipped with automatic sampler, Agilent 1100 LC chemical workstation, G1311 quaternary gradient pump and VWD detector from Agilent technologies Co., Ltd.; Thermo water distiller from Thermo fisher scientific Co., Ltd.; ZRD-A5110 electric oven thermostat blast from Shanghai Zhicheng Analytical Instruments Co., Ltd.; FA2204B electronic analytical balance from Shanghai Jinghai Instrument Co., Ltd.; UV-1800 ultraviolet visible spectrophotometer from

Beijing sharp Analytical Instrument Co., Ltd.; DHY-300 pulverizer from Beijing Donghua Medical Equipment Co., Ltd.; LI-6400XT photosynthetic instruments from American LI-COR company.

Reagent : Pure acetonitrile and methanol were chromatography pure, phosphoric acid, acetic acid, ethanol and acetone were analytically pure, ultra pure water, 0.1 mol/L HCl was prepared by 36% hydrochloric acid, catalpol and verbascoside standard was purchased from Chinese food and drug Inspection Institute (Batch No.110808-201210 and 111530-201310).

Per plant fresh weight : Weight of six plant varieties were estimated in Xia Zhuang Village, Bei Guo Town before *Rehmannia* harvesting, Three sites were selected for each variety and 10 plants and in succession from each site, 30 plants of each varieties were selected for weight determination and the average value was determined.

Yield : Yield of six varieties was estimated at three spots in Wuzhi and the value was converted to yield per hectare and the average value was determined.

Determination of index components

Determination of catalpol content : Standard solution preparation: 11.4 mg catalpol was weighed and put into a 10 ml volumetric flask, cetonitrile-0.1%, phosphoric acid (1:99) solution was added to the volume.

Linear relationship : 0.5, 1, 1.5, 2, 2.5 mL standard solution were respectively transferred to 5 mL volumetric flasks, dissolved by mobile phase to constant volume, different concentration of catalpol standard solution were prepared and the peak area of catalpol standard with different concentrations was determined according to the given chromatographic conditions.

Precision test : Five successive injections of the same standard solution were conducted according to the given chromatographic conditions for the determination of peak area. RSD value was 0.20%, that showed that the precision of the instrument is fine.

Chromatography conditions : sample preparation and determination method were referred to the 2010 edition of the Chinese pharmacopoeia for the determination of the content of *Rehmannia* (Pharmacopoeia Committee of the Ministry of health of the People's Republic of China 2010).

Determination of verbascoside content : Preparation of the standard solution: 9 mg of Verbascoside was weighed and put into 25 ml capacity bottle, acetonitrile-0.1%; acetic acid solution (16:84) mobile phase was added to the volume.

Linear relationship : 1, 2, 4, 6, 8 ml standard solution were respectively transferred to 10 ml volumetric flasks, dissolved by mobile phase and different concentrations of verbascoside standard solution were prepared, then they were analyzed according to the given chromatographic conditions.

Precision test : Five successive injections of the same standard solution were conducted according to the given chromatographic conditions for the determination of peak area. RSD value of 0.97% showed the precision of the instrument is fine. Chromatography conditions, sample preparation and determination method were referred to the 2010 edition of the Chinese pharmacopoeia for the determination of the content of *Rehmannia*.

Resistance identification : The *Septoria digitalis* Pass and identification of leaf ring rot resistance of new varieties of *Rehmannia* were identified by field investigation. The investigation was conducted in September when the leaf disease was serious. Three sites were selected for each variety and 10 plants were selected in rows from each site. Leaves more than 5 cm were selected randomly and investigated, the disease severity was recorded, disease index was counted, the relative resistance index was calculated, finally, identification of resistance was conducted.

Determination of chlorophyll and anthocyanin content : Five plants of each variety were selected randomly, three matured leaves of the second round of each plant were selected, washed and dried. The main veins were removed and the rest were cut into pieces. Chlorophyll and anthocyanin content were determined following the method of Li *et al.* (2007), and each variety were repeated for five times.

Determination of photosynthetic characteristics : Photosynthetic characteristics were measured from 09:30 to 11:30 a.m. in July 29, 2015. Five matured leaves of the second round from the top of the plant were randomly selected and photosynthetic rate (Pn), stomatal conductance (Cond), transpiration rate (Tr) and intercellular CO² concentration (Ci) were determined by 6400XT.

Results and Discussion

Yield of different varieties : The result of per plant fresh weight of six *Rehmannia* varieties is shown in Fig. 1 and the yield of six varieties in different spots is shown in Table 1.

The weight of different *Rehmannia* varieties is as follows: Huaidi 81>85-5>Golden Nine>Huaifeng>Qinhuai>Beijing No. 3. There was extremely significant differences between Huaidi 81 and five main varieties, which proved that Huaidi 81 had extremely significant advantages in terms of per plant fresh weight.

The yield results in 3 spots showed that the yield in 3 spots and average yield of Huaidi 81 were higher than the main varieties. The average yield of Huaidi 81 was higher than 85-5 variety by 29.7%, higher than Golden Nine by 30.2%, higher than Huaifeng by 33.2%, higher than Qinhuai by 39.5%, higher than Beijing No.3 by 47%. Yield determination results showed that Huaidi 81 had significant advantage in terms of yield.

Index components determination of different *Rehmannia* varieties : Catalpol standard and samples were tested according to the Pharmacopoeia method. Different concentrations of catalpol standard were investigated for linear relationship, the linear regression equation was $Y=1.9967X-45.21$, $R^2=0.9999$, which indicated that catalpol had good linear relationship from 114 to 570 $\mu\text{g ml}^{-1}$. The catalpol content of different *Rehmannia* varieties is shown in Table 2. Verbascoside standard and samples were tested according to the Pharmacopoeia method. Different concentrations of Verbascoside standard were investigated for linear relationship. The linear regression equation was $Y=52.578X-39.223$, $R^2=0.9999$, which indicated that verbascoside had good linear relationship from 36 to 288 $\mu\text{g ml}^{-1}$. The verbascoside content of different *Rehmannia* varieties is shown in Table 2.

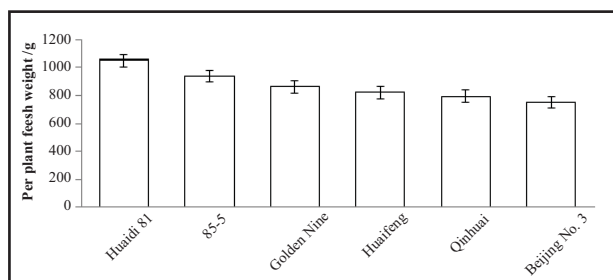


Fig. 1 : Per plant fresh weight of different *Rehmannia* varieties

Table 1 : Yield of different varieties in different spots in 2013

Varieties	Yield of Xia Zhuang Village, Bei, Guo Town (kg.hm ⁻²)	Yield of Guo Village, Chengguan Town (kg.hm ⁻²)	Yield of Zhong Situ Village, Da Hongqiao Town (kg.hm ⁻²)	Average yield of three spots (kg.hm ⁻²)	Yield increase of Huaidi 81 compared with the main varieties (%)
Huaidi 81	81997.0	82177.6	82885.1	82353.7	—
85-5	62944.8	62589.6	64889.6	63474.6	29.7
Golden Nine	62788.1	63207.5	63709.0	63234.3	30.2
Huaifeng	61603.0	61301.5	62616.4	61840.3	33.2
Qinhuai	58919.4	58283.6	59962.7	59055.2	39.5
Beijing No.3	56198.5	55110.4	56801.5	56037.3	47.0

Table 2 shows that catalpol content of six *Rehmannia* varieties in the following order : Beijing No.3 (1.601%)> Qinhuai (1.588%)> Huaidi 81 (1.314%)> Golden Nine > 85-5 (1.073%)> Huaifeng(0.924%). The catalpol content of Huaidi 81 was lower than that of Beijing No. 3 and Qin Huai, but higher than that of Golden Nine, 85-5 and Huaifeng. Analysis by SPSS software showed that there was no significant difference in the catalpol content between Huaidi 81 and Golden Nine, but extremely significant difference between Huaidi 81 and other varieties was observed. Its content was much higher than the standard in 2010 China Pharmacopoeia and the content of catalpol in *Rehmannia* must not less than 0.20%, which indicated that the index components of catalpol content of Huaidi 81 had comparative advantage over the main varieties. Verbascoside content of six *Rehmannia* varieties were 0.022 to 0.096%, they ranked in the following order: Huaidi 81 (0.096%) > Qinhuai (0.069%)> 85-5 (0.047%) > Beijing No.3 (0.035%) > Huaifeng (0.023%) > Golden Nine (0.022%). The verbascoside content of Huaidi 81 was more than five main cultivars. There was extremely significant difference between Huaidi 81 and other varieties.

Resistance identification : The resistance identification were conducted in the new variety and the main cultivars according to the methods above, the results were shown in Table 3.

Table 3 shows that Huaidi 81 showed high resistance to *Septoria digitalis* Pass, 85-5, Huaifeng and Golden Nine had moderate resistance to *Septoria digitalis* Pass, Beijing No.3 was moderately susceptible to *Septoria digitalis* Pass, Qinhuai was highly susceptible to *Septoria digitalis* Pass; Huaidi 81, Beijing No.3 and Golden Nine had moderate resistance to leaf ring rot, 85-5 and Qinhuai were moderately susceptible to leaf ring rot, Huaifeng was highly susceptible to leaf ring rot. Huaidi 81 showed high resistance to *Septoria*

digitalis Pass and middle resistance to leaf ring rot, Huaidi 81 showed good resistance to leaf diseases.

Determination of chlorophyll and anthocyanin : The chlorophyll and anthocyanin content in different varieties were determined and the results are shown in Table 4. The chlorophyll content of different *Rehmannia* varieties were 2.51 to 2.71 mg g⁻¹, they ranked in the following order: Huaidi 81 (2.71 mg g⁻¹) > 85-5 (2.69 mg g⁻¹) > Golden Nine (2.66 mg g⁻¹) > Huaifeng (2.56 mg g⁻¹) > Qinhuai (2.52 mg g⁻¹) > Beijing No.3 (2.51 mg g⁻¹). Chlorophyll content of Huaidi 81 was higher than that of five main varieties. There was insignificant difference between Huaidi 81, 85-5 and Golden Nine, while significant difference was found between Huaidi 81 and other three varieties, which indicated that Huaidi 81 had significant advantage in terms of chlorophyll content. The relative anthocyanin concentration were 0.087 to 0.169. They ranked as follows: Golden Nine(0.169)> Beijing No.3 (0.136)> Huaidi 81 (0.105)> Huaifeng (0.103)> Qinhuai (0.097)> 85-5 (0.087). Anthocyanin content of Huaidi 81 was lower than Golden Nine and Beijing No.3 and the difference was significant. Anthocyanin content of Huaidi 81 was higher than Huaifeng, Qinhuai and 85-5. There was insignificant difference between Huaidi 81 and Huaifeng, while significant difference between Huaidi 81 and Qinhuai. High significance between Huaidi 81 and 85-5, indicated that the anthocyanin content of Huaidi 81 had relative advantage.

Table 2 : Catalpol and verbascoside content of different *Rehmannia* varieties

Varieties	Catalpol content %	Verbascoside content %
Huaidi 81	1.314±0.02 ^{bb}	0.096±0.005 ^{aa}
85-5	1.073±0.02 ^{cc}	0.047±0.001 ^{cc}
Golden Nine	1.277±0.02 ^{bb}	0.022±0.001 ^{cc}
Huaifeng	0.924±0.06 ^{dd}	0.023±0.002 ^{cc}
Qinhuai	1.588±0.01 ^{aa}	0.069±0.001 ^{bb}
Beijing No.3	1.601±0.03 ^{aa}	0.035±0.001 ^{dd}

Table 3 : Resistance identification of different *Rehmannia* varieties (n=30)

	Septoria digitalis pass			Leaf ring rot		
	Disease index	Resistance index	Level	Disease index	Resistance index	Level
Huaidi 81	0.14	0.86	HR	0.35	0.65	MR
85-5	0.38	0.62	MR	0.59	0.41	MS
Beijing No.3	0.44	0.56	MS	0.36	0.64	MR
Qinhuai	—	—	HS	0.43	0.57	MS
Huafeng	0.37	0.63	MR	—	—	HS
Golden Nine	0.33	0.67	MR	0.40	0.60	MR

Determination results of photosynthetic characteristics :

Table 5 showed that the photosynthetic rate of different varieties were 1.83 to 2.41 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Photosynthetic rate of Huaidi 81 was higher than that of other varieties, and the differences between Huaidi 81 and other varieties were extremely significant. The stomatal conductance of different varieties were 0.048 to 0.372 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Stomatal conductance of Huaidi 81 was lower than that of Beijing No.3, and the difference was extremely significant. The transpiration rate of different varieties were 1.99 to 5.38 $\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Transpiration rate of Huaidi 81 was lower than that of Beijing No.3, while difference between them was insignificant. Transpiration rate of Huaidi 81 was higher than other varieties, and differences between Huaidi 81 and other varieties were extremely significant. The intercellular CO_2 concentration of different varieties were 367.96 to 334.48 $\mu\text{l l}^{-1}$. The intercellular CO_2 concentration of Huaidi 81 was lower than other main cultivars, and the difference was extremely significant.

The photosynthetic index results showed that the photosynthetic rate of Huaidi 81 was higher than that of the main cultivars. The transpiration rate and stomatal conductance was higher and the intercellular CO_2 concentration was lower. The net photosynthetic rate of plants directly reflects the ability of photosynthesis (Hou *et al.*, 2009), this illustrates that Huaidi 81 had good photosynthetic capacity which was suitable for its high yield.

Correlation analysis of photosynthetic rate with chlorophyll and anthocyanin : The photosynthetic rate and chlorophyll content were analyzed by SPSS software. Chlorophyll showed significant positive correlation with photosynthetic rate with correlation coefficient of 0.820. Anthocyanin does belong to photosynthetic pigment, it was not directly involved in photosynthesis, but effects the photosynthetic rate (Kytridis *et al.*, 2008). Studies have shown that anthocyanins play a certain role in the defense and

mitigation of photooxidative damage in plants under low temperature, drought, salinity and other stress conditions (Wang *et al.*, 2012). Correlation between photosynthetic rate and chlorophyll content in six varieties is shown in Fig. 2.

Chlorophyll and anthocyanin content showed that the chlorophyll content of Huaidi 81 was higher than that of the main varieties. The anthocyanin content of Huaidi 81 was moderate. Chlorophyll content reflects the intensity of photosynthesis (Wei *et al.*, 1994). The chlorophyll content was positively correlated with photosynthetic rate in a certain range (Chen *et al.*, 2010). The results showed that the chlorophyll content in six varieties was positively related to the photosynthetic rate and correlation reached a significant level, which corresponds with reported studies. Anthocyanin does not belong to photosynthetic pigment, but it can regulate the photon absorption of chlorophyll. It has the function of filtering, attenuation and reflection of light on the blade. Therefore, anthocyanin content of *Rehmannia* can reflect the intensity of photosynthesis to a certain extent (Wang *et al.*, 2012).

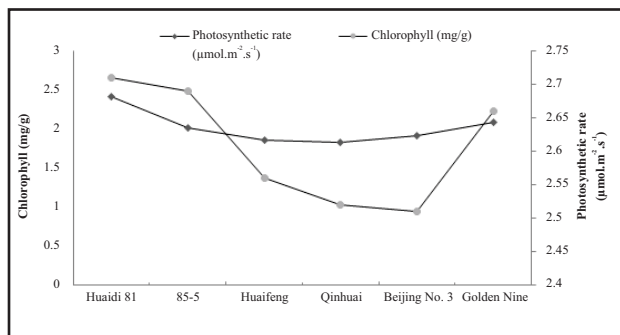
There are differences in the yield and index components of *Rehmannia* due to different varieties or different growth environment (Li *et al.*, 2011; Li *et al.*, 2012). In the present study, different varieties were planted on the same experimental plot to reduce the influence of other

Table 4 : Chlorophyll and anthocyanin content in different *Rehmannia* varieties

Varieties	Chlorophyll content	Relative concentration of anthocyanin
Huaidi 81	2.71±0.011aA	0.105±0.0058cC
85-5	2.69±0.038aA	0.087±0.0079eD
Huafeng	2.56±0.030bB	0.103±0.0034cdC
Qinhuai	2.52±0.028bcB	0.097±0.0058dCD
Beijing No.3	2.51±0.025cB	0.136±0.0026bB
Golden Nine	2.66±0.021aA	0.169±0.0038aA

Table 5 : Photosynthetic characteristics determination results of different *Rehmannia* varieties

Varieties	Photosynthetic rate ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	Soma conductance ($\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	Transpiration rate ($\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	Intercellular CO_2 concentration ($\mu\text{L}\cdot\text{L}^{-1}$)
Huaidi 81	2.41±0.048aa	0.290±0.011 ^{bb}	4.31±0.67 ^{aa}	334.48±2.51 ^{cd}
85-5	2.02±0.047bcb	0.151±0.014 ^{cc}	2.70±0.62 ^{bb}	358.72±0.97 ^{cb}
Huairfeng	1.85±0.070cb	0.061±0.013 ^{dd}	2.36±0.80 ^{bb}	362.88±1.09 ^{bab}
Qinhuai	1.83±0.051cb	0.048±0.008 ^{dd}	1.99±0.72 ^{bb}	362.72±1.96 ^{bab}
Beijing No.3	1.91±0.113bcb	0.372±0.074 ^{aa}	5.38±0.89 ^{aa}	346.91±2.50 ^{dc}
Golden Nine	2.08±0.081bb	0.197±0.010 ^{cc}	2.79±0.24 ^{bb}	367.96±1.60 ^{aa}

**Fig. 2** : The correlation between photosynthetic rate and chlorophyll of different rehmannia varieties

factors on the determination of results. The determination of Chlorophyll and Anthocyanin of different *Rehmannia* varieties was also carried out in the same experimental plot. This could reflect the differences between varieties more accurately. Per plant fresh weight and yield of Huaidi 81 had significant advantage over the main varieties, the index component of Verbascoside content of Huaidi 81 had significant advantages and the catalpol content of Huaidi 81 had comparative advantage. The index component is much higher than the standard in Chinese Pharmacopoeia. Huaidi 81 showed good resistance to leaf diseases and higher chlorophyll content than the main varieties and also revealed moderate anthocyanin content. Moreover, photosynthetic rate of Huaidi 81 was higher than the main varieties. For the excellent quality of Huaidi 81, it was identified as a new *Rehmannia* variety on December 18, 2014 and it can be popularized and applied in Huai area and other areas having similar environmental conditions.

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