**Distinctive morphological characteristics contribute to the identification of
Artemisia annua L. germplasms with high yield and high artemisinin content**

Jin-e Fu¹, Li Feng^{2*}, Shu-gen Wei^{1,2*}, Xiao-jun Ma^{1,2}, Rong-shao Huang³, Shi-xin
Feng¹, Qing-song Dong¹, Zhi-gang Yan^{1,2}

1. Guangxi branch of Institute of Medical Plants Development, Chinese Academy of
Medical Science & Peking Union Medical College, Nanning 530023, P. R. of
China
2. Institute of Medicinal Plants Development, Chinese Academy of Medical Sciences
& Peking Union Medical College, Beijing 100093, P. R. of China
3. Agriculture college of Guangxi university, Nanning 530005, P. R. of China

*Corresponding authors

Li Feng, P.h.D

Institute of Medicinal Plant Development,

Chinese Academy of Medical sciences & Peking Union Medical College

No.151, Malianwa North Road, Haidian District, Beijing 100193, P. R. of China.

TEL: +86-010-57833468; FAX: +86-010-57833468

E-mail: FengL_2014@hotmail.com

Shu-gen Wei, P.h.D

Guangxi branch of Institute of Medical Plants Development, Chinese Academy of

Medical Science & Peking Union Medical College, Nanning 530023, P.R. of China

No.189 Changgang Road, Nanning, Xingning District 530023, P. R. of China.

TEL: +86-0771-5602461; FAX: +86-0771-5602461

E-mail: weishugen2@163.com

Abstract

Artemisia annua L., a famous traditional Chinese medicine, has been used as an antimalarial herbal medicine for thousands of years. Artemisinin, main bioactive constituent of *Artemisia annua* L., is the principal contributor to its pharmacological effects. Previous studies have shown that *Artemisia annua* L. of different germplasm has different morphological characteristics, herb yield, and artemisinin content. We propose a hypothesis that the morphological characteristic of *Artemisia annua* L. may contribute to breeding new cultivars with high yield and high artemisinin content. To test this hypothesis, 8 morphological characteristics (plant height, stem bottom diameter, crown diameter, distance of internode, the number of primary branches, primary branch angle, leaf length, and leaf width) of *Artemisia annua* L. from 64 germplasm resources were studied. The correlations between morphological characteristics and herb yield, artemisinin content were studied by using partial least squares regression (PLSR) analysis, correlation analysis, and path analysis. The results indicated that: 1). The same characteristic of different germplasm resources varied greatly under the same cultivation and management condition; 2). Both the artemisinin content and herb yield were positively correlated to plant height, stem bottom diameter, crown diameter, distance of internode, leaf width, and negatively correlated to the number of primary branches. Nevertheless, the coefficient of correlation are very low and varying between $r = 0.25$ to $r = 0.46$; 3). The strongest relations were found for plant height and stem bottom diameters. In conclusion, both

plant height and stem bottom diameter had the most important positive influence on artemisinin content of the leaves and herb yield. The results of the present study can help to increase breeding efficiency for getting new *Artemisia annua* L. cultivars with high herbal yield and high artemisinin content.

Keyword: *Artemisia annua* L., artemisinin, breeding new germplasm, morphological characteristic

Introduction

Artemisia annua L. named Huanghuahao in Chinese, is an annual herb of Asteraceae *Artemisia* and the major source of artemisinin. Artemisinin, the main bioactive component of *Artemisia annua* L. has been used to treat malaria for thousands of years. According to WHO reports, there is about 4-5 hundred million people infected malaria in more than 100 countries all over the world. One to two million patients died from malaria every year, especially in Africa, Southeast Asia and Latin America (Murray *et al.*, 2012).

Although *Artemisia annua* L. distributes widely in the world, the artemisinin content varies greatly among herbs from different places and germplasm. It is well known that the industrial extraction cost significantly reduces with the artemisinin content increase. Moreover, the annual demand for artemisinin is very big all over the world.

Recently, studies of *Artemisia annua* L. mainly focus on wild acclimation, resources investigation, characters description, breeding activities, planting technology, DNA polymorphism analysis and so on (Carlen and Simonnet, 2015; Chengqiong and Xiuhong, 1999; Delabays *et al.*, 2001; Elhag *et al.*, 1992; Liu *et al.*, 1998; Özgüven *et al.*, 2008; Ram *et al.*, 1997; Simonnet *et al.*, 2008). Especially in 2010, Ian A.*et al* (Graham *et al.*, 2010b) identified the gene loci which affects the artemisinin content. In some other plants, the relationship between characteristics and

herb yield or active component content has already been noticed and studied (Thippeswamy *et al.*, 2003).

Therefore, it is crucial to clarify the relationship between morphological characteristics and herbal yield, artemisinin content of *Artemisia annua* L.. Breeding germplasm with high artemisinin content and high yield not only can reduce the extraction cost but also contribute to the quality control of the herb. To the best of our knowledge, there was few study has been conducted to examine the relationship between morphological characteristics and herbal yield, artemisinin content of *Artemisia annua* L..

In the present research, 64 germplasms (53 from Guangxi province, 5 from Hunan province, 3 from Sichuan province, 3 from Shanxi, Guangdong, and Jiangsu province respectively, China) of *Artemisia annua* L. were collected, established, and analyzed by studying the characteristics of stem, leave and branch system, leaf yield, and artemisinin content in the leaves. The relations of these parameters could provide scientific evidence for breeding quicker high yielding and high artemisinin-containing new cultivars.

1 Materials and methods

In this study, *Artemisia annua* L. germplasm resources were collected from 63 different places of China and planted at Natang village of Jingxi county, Guangxi province, China. *Artemisia annua* L. germplasm was identified by professor Xiao-jun, Ma, Institute of Medicinal Plants Development, Peking Union Medical College & Chinese Academy of Medical Sciences. Voucher specimens were preserved in

Guangxi branch of Institute of Medical Plants Development, Chinese Academy of Medical Science & Peking Union Medical College.

1. 1 Designs of field trial

The random block design with 3 replications was used in the experiment. The plot size was 10 m² and the planting density was 3 plants/m². Seeds were sowed on February 25th, 2014. Thirty days after sprouting, the herbs were watered with 2% ammonium carbonate water solution. Then, the herbs were fertilized with compound fertilizer (N:P:K = 15%:15%:15%) at a rate of 150 kg·ha⁻¹ one month later. Other management measures followed traditional cultivation. The weeding was conducted manually when necessary.

1. 2 Characteristics observation

Twenty plants of each germplasm resource were collected at random for morphological observation. Eight characteristics including plant height, stem base diameter, crown diameter, the number of primary branches, primary branch angle, internode length, leaf length, and leaf width were observed. The length and width of leaves were measured by a ruler with a minimum scale is a millimeter. The primary branch angle was gauged by an angle meter with a minimum scale is one degree.

1. 3 Determination of artemisinin content

The extraction of artemisinin was performed as previously described with minor modifications (*Hao et al., 2002*). Briefly, raw herb material was dried to constant weight at 40 °C and sifted through a 60 mesh sieve. One gram sifted raw material was placed in a 100 ml Erlenmeyer flask and stuffed with a rubber stopper. Ultrasonic of

75 Hz was used to the extraction procedure. Forty milliliters of 30 ~ 60 °C petroleum ether was added to each aliquot. The extraction was repeated 3 times with 20 min each time. The extract was combined and filtered. Artemisinin content was detected by a Shimadzu 1206 UV spectrophotometer (Japan) and measured in triplicate at 292 nm by using a previously published method (Chunli, 2007). The three measured values were averaged. All solvents used were analytical grade and ultra-pure water was self-made with a Milli-Q[®] system.

1. 4 Processing and Statistical analysis of data

Data were analyzed by SPSS 19.0 (IBM), SIMCA-P software (version 11.5, Umetrics, Sweden), and R programming language. For path analysis, R package "agricolae" was used (De Mendiburu, 2009).

2 Result and Discussion

2. 1 Character variance analysis

Plant height, stem base diameter, crown diameter, yield, and artemisinin content of 20 plants derived from the 64 germplasm resources were determined in the budding stage in the first twenty days of August of 2014. The results were shown in table 1. From table 1, it can be seen that although cultivated in the same condition, the variances of the selected characteristics were large. However, coefficient of variation of yield and artemisinin content were larger than these characteristics.

Insert Table 1

2. 2 Partial least squares regression (PLSR) analysis of the 64 germplasm resources

PLSR analysis of the 64 germplasm resources was performed by using SIMCA-P software. In PLSR analysis, the observations (Fig. 1A) are the 64 germplasm resources and the variables (Fig. 1B) are the 8 morphological characteristics (X variable), artemisinin content (Y1 variable), and yield (Y2 variable). In scores plot of PLSR analysis (Fig. 1A), nearby observations (germplasm resources) have similar morphological characteristics, artemisinin content, and yield. It also can be seen that germplasm originated from Shanxi and Jiangsu province have the largest separation with other germplasm resources. Some outliers (observations outside the Hotelling ellipse) in germplasm from Guangxi province were also observed. From Fig. 1B, it can be seen that yield and plant height has a larger correlation. Artemisinin content and stem bottom diameter, leaf width, leaf length, distance of internode, and crown diameter have larger correlations. Primary branch angle has a negative effect (x coordinate below 0) on both the yield and the artemisinin content.

Insert Fig. 1.

Insert Table 2

2.3 Correlation analysis of main characteristics

As is shown in table 2, the yield was positively correlated to plant height, stem base diameter, crown diameter, internode length and the number of primary branches and negatively correlated with primary branch angle. The correlation coefficient between yield and stem base diameter was the biggest (0.46). Artemisinin content was positively related to plant height, stem base diameter, crown diameter, internode length, leaf length, and negatively related to primary branch angle and the number of

primary branches. The correlation coefficient between artemisinin content and stem base diameter was the biggest (0.27).

Insert Table 3 and Table 4

2. 4 Path analysis of major characteristics on herb yield and artemisinin content

As is shown in table 3, characteristics which have a direct positive influence on artemisinin content were leaf width, plant height, and stem bottom diameter. The correlation coefficients were 0.55, 0.52, and 0.12 respectively. Crown diameter, distance of internode, the number of primary branches, primary branch angle, and leaf length had a direct negative correlation with artemisinin content. The corresponding correlation coefficients were -0.18, -0.24, -0.53, -0.15, and -0.58 respectively. Plant height, primary branch angle, and leaf length had negative effects on artemisinin content through other characteristics (total indirect effects). Stem bottom diameter, crown diameter, distance of internode, the number of primary branches, and leaf length had positive effects on artemisinin content through other characteristics (total indirect effects)

As indicates in table 4, the characteristics which have a direct positive influence on yield were plant height, stem bottom diameter, distance of internode, the number of primary branches. The corresponding correlation coefficients were 0.34, 0.37, 0.19, 0.06, and 0.04 respectively. The characteristics which have a direct negative influence on artemisinin content were leaf width and crown diameter. The corresponding correlation coefficients were -0.05 and -0.23 respectively.

3. Discussion

Artemisia annua L. has good adaptability and distributed widely in the world. Nevertheless, the yield and the artemisinin content in herbs of different source varied greatly due to polymorphism of genetic traits and the difference of the growth condition (Sangwan et al., 1999). Thus, it is of critical importance to breeding cultivars with high yield and high artemisinin content and provide high-quality raw materials for the industrial production of artemisinin. Various method has been applied to assess the quality of the herb, such as path analysis (Lihua, 2007), correlation analysis (Ma et al., 2008), grey related degree and cluster analysis (LiYing et al., 2009), and genetic study (Graham et al., 2010a). The main aim of these studies was to clarify the relationship between yield, artemisinin content and growth environment, morphological characteristics, and genetic factors.

Improving the yield and the artemisinin content is the main aim of the breeding of this herb. Thus, it is essential to clarify factors that affect the yield and the artemisinin content. The result of this study can accelerate the breeding process and also provide scientific suggestions for other medical plants' breeding.

In the present study, 64 *Artemisia annua* L. germplasm resources derived from three main producing areas (Guangxi, Guangdong, and Sichuan) and two poor-quality areas (Shanxi and Jiangsu) of this herb in China were collected and planted in the same environment. Eight major morphological traits of this herb were determined to find the potential correlation between these traits and the yield, artemisinin content.

From table 1, it can be seen that the variances of the 8 major traits, the yield, and the artemisinin content of herbs from different germplasm are great under the same

growth condition. This indicated the genetic variation of different germplasms were large under the same cultivation and management conditions.

In order to improve the breeding efficiency, speed up the breeding rate, and reveal the effects of each characteristic on the artemisinin content and herb yield, PLSR, correlation analysis and path analysis were performed in this research.

Partial least squares regression analysis is a multivariate statistical method and mainly used to study the relations between response variable (Y variable) and independent variable (X variable) and to observe the potential clustering trends among different observations. PLSR has been used in the quality control of food (Martens and Martens, 2000) and herbal medicine (Li et al., 2013). In this study, the germplasm of Shanxi and Jiangsu separated greatly with other germplasm (Fig. 1A). This indicates there are notable differences between the two germplasm and other germplasm concerning the 8 morphological characteristics, yield, and artemisinin content. Primary branch angle, plant height, and yield, and the number of primary branch are the major characteristics contribute to the separation because of their larger loadings value (distance to the origin) (Fig. 1B). The loadings plot of the PLSR (Fig. 1B) also indicates that plant height are the major influence factor of the yield and stem bottom diameter, leaf width, leaf length, distance of internode, and crown diameter are the main influence factors of artemisinin content. Primary branch angle has a negative effect (x coordinate is negative) on both the yield and the artemisinin content.

Correlation analysis is commonly used to study the relations between two variables. The correlation coefficient is the reflection of the correlation of the two

variables. The results of the correlation analysis indicated that there were some correlations between the characteristics and yield, artemisinin contents (Table 2). The yield and artemisinin content were both positively related to plant height, stem bottom diameter, crown diameter, distance of internode, leaf length, and leaf width and negatively correlated to primary branch angle. The artemisinin content was also negatively related to the number of primary branch. The plant height ($r = 0.45$), stem bottom diameter ($r = 0.46$), crown diameter ($r = 0.42$), distance of internode ($r = 0.39$), and the number of primary branch ($r = 0.34$) had a larger correlation with the yield and can be considered as the major influence factors of the yield. The plant height ($r = 0.25$), stem bottom diameter ($r = 0.27$), distance of internode ($r = 0.22$), primary branch angle ($r = -0.22$), and leaf width ($r = 0.25$) had a larger correlation with the artemisinin content and can be considered as the main influence factors of the artemisinin content. Besides, we observed that plants with high artemisinin content often growing luxuriant, with large and sturdy of stem, long distance of internode, wider leaves, fewer primary branches and small primary branch angle, which were consistent with results of Elhag's (Elhag *et al.*, 1992).

Path analysis is a form of multiple regression methods and mainly used to study the complex relation between multiple independent variables and dependent variables (Garson, 2008). In path analysis, the correlation coefficient was decomposed into direct correlations (direct effects) and correlations through other variables (indirect effects). Path analysis can provide a deep insight into the relationship between the independent variables and dependent variables and was widely used in various fields

including breeding (Güler et al., 2001).

From table 3 and 4, it can be seen that the total correlation coefficients between the morphological characteristics and the yield and artemisinin content are the same as table 2 but decomposed into direct effects and a series of indirect effects. From table 3, it can be seen that plant height and stem bottom diameter had larger direct positive effects on the yield. The influence of crown diameter, distance of internode, and the number of primary branches on the yield were mainly mediated in an indirect way. This reminds us plant height and stem bottom diameter should be considered as the major influence factors concerning the herb yield. From table 4, it can be seen that plant height and leaf width had large direct positive effects and also indirect negative effects on the artemisinin content through other characteristics. The influences of crown diameter, distance of internode, and the number of primary branches on the artemisinin content were mainly mediated in an indirect way. Leaf width had almost equally positive and negative influence on the artemisinin content. Both the direct and the indirect effect of primary branch angle on the artemisinin content were negative. So, plant height, stem bottom diameter, and leaf width should be considered as the major positive influence factors and primary branch angle can be considered as major negative influence factor of artemisinin content.

4. Conclusion

In summary, both herb yield and artemisinin content were positively correlated to plant height and stem bottom diameter and negatively correlated to primary branch angle. Plant height, stem bottom diameter, and primary branch angle are the main

characteristics that contribute to the identification high-value *Artemisia annua* L. germplasm.

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Table 1

Results of the 8 morphological characteristics, leaf dry yield, and artemisinin content of the leaves of the 64 germplasm (n = 1280)

Traits (unit)	Maximum	Minimum	Mean $\pm$ S.D.	C.V. (%)
Primary branch angle ($^{\circ}$)	60.4	42.0	48.4 ± 3.5	7.1
The number of primary branches	85.0	49.0	71.0 ± 7.0	10.0
Crown diameter (cm^2)	120.5	62.6	104.1 ± 11.3	10.9
Distance of internode (cm)	4.8	2.5	3.6 ± 0.4	11.3
Plant height (cm)	246.8	123.7	209.8 ± 23.7	11.3
Leaf length (cm)	9.0	4.9	7.5 ± 0.9	11.4
Leaf width (cm)	8.4	4.1	6.7 ± 0.9	13.0
Stem bottom diameter (cm)	4.7	1.5	2.4 ± 0.4	17.8
Artemisinin content (g/g)	1.0	0.2	0.6 ± 0.2	25.2
Yield (g/m ²)	123.1	597.0	355.3 ± 93.2	26.2

S.D., standard deviation; C.V., coefficient of variation.

Table 2Results of correlation analysis of the 8 characteristics and yield, artemisinin content ^a

Major traits	Plant height (cm)	Stem bottom diameter (cm)	Crown diameter (cm)	Distance of internode (cm)	The number of primary branches	Primary branch angle (°)	Leaf length (cm)	Leaf width (cm)
Yield (g/m ²)	0.45 ^{**}	0.46 ^{**}	0.42 ^{**}	0.39 ^{**}	0.34 ^{**}	-0.06	0.02	0.11
Artemisinin content (g/g)	0.25 [*]	0.27 [*]	0.18	0.22	-0.03	-0.22	0.10	0.25 [*]

Note: a, values are correlation coefficients; * correlation is significant at 0.05 level; ** correlation is significant at 0.01 level;

Table 3Results of path analysis of the 7 characteristics and yield of *Artemisia annua* L.^a

Major traits	Indirect effects							Total indirect effects	Direct effects	Total correlation coefficients
	→X1	→X2	→X3	→X4	→X5	→X6	→X8			
Plant height (X1)		0.11	-0.05	0.13	0.03	-0.01	-0.10	0.11	0.34	0.45
Stem bottom diameter (X2)	0.10		-0.01	0.07	0.01	-0.01	-0.07	0.09	0.37	0.46
Crown diameter (X3)	0.31	0.10		0.11	0.03	0.00	-0.09	0.47	-0.05	0.42
Distance of internode (X4)	0.22	0.14	-0.03		0.01	-0.01	-0.13	0.20	0.19	0.39
The number of primary branches (X5)	0.19	0.09	-0.03	0.03		0.01	0.00	0.28	0.06	0.34
Primary branch angle (X6)	-0.05	-0.05	0.00	-0.07	0.01		0.07	-0.10	0.04	-0.06
Leaf width (X8)	0.15	0.12	-0.02	0.11	0.00	-0.01		0.34	-0.23	0.11

Note: a, because the correlation between leaf length and yield is too small (0.02), the software neglected this characteristic when conducted path analysis; "+"; positive correlation; "-" negative correlation.

Table 4

Results of path analysis of the 8 characteristics and artemisinin content

Major traits	Indirect effects									Total indirect effects	Direct Effects	Total correlation coefficients
	→X1	→X2	→X3	→X4	→X5	→X6	→X7	→X8	→X9			
Plant height (X1)		0.03	-0.16	-0.16	-0.29	0.02	-0.14	0.24	0.19	-0.27	0.52	0.25
Stem bottom diameter (X2)	0.15		-0.05	-0.09	-0.12	0.02	-0.12	0.18	0.19	0.15	0.12	0.27
Crown diameter (X3)	0.48	0.03		-0.14	-0.31	0.01	-0.09	0.22	0.17	0.36	-0.18	0.18
Distance of internode (X4)	0.34	0.04	-0.10		-0.09	0.05	-0.25	0.30	0.16	0.46	-0.24	0.22
The number of primary branches (X5)	0.29	0.03	-0.10	-0.04		-0.02	0.21	0.00	0.14	0.50	-0.53	-0.03
Primary branch angle (X6)	-0.08	-0.02	0.01	0.09	-0.08		0.20	-0.16	-0.02	-0.07	-0.15	-0.22
Leaf length (X7)	0.12	0.02	-0.03	-0.10	0.19	0.05		0.42	0.01	0.68	-0.58	0.10
Leaf width (X8)	0.22	0.04	-0.07	-0.14	0.00	0.04	-0.45		0.04	-0.30	0.55	0.25

"+" , positive correlation; "-" negative correlation.

Fig. 1. Partial least squares regression (PLSR) analysis scores plot (A) and loadings plot (B) of the 64 germplasm resources.