Research Paper

Quality Improvement of Artificial Cultivation of *Anoectochilus Roxburghii*

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Abstract

This study observes the artificial cultivation process for *Anoectochilus roxburghii* conducted by L. Company. The purpose of this study is to find out factors which are responsible for the cultivation of high quality *Anoectochilus roxburghii* through analysing the collected data using statistical methods. By investigating the different cultivation environments of *Anoectochilus roxburghii*, this study concludes that *Anoectochilus roxburghii* produced in Fujian Province has a better quality than those produced elsewhere. Furthermore, detailed analysis shows that the cultivation environment should meet the following conditions in order to obtain high-quality *Anoectochilus roxburghii*: medium MS, humus soil (600), the river sand (200) and perlite (200). Future work is required to prove the validity of this research result in the efficiency and quality improvement of *Anoectochilus roxburghii* cultivation.

Keywords: *Anoectochilus Roxburghii*, Artificial Cultivation, Rank Sum Test, Orthogonal Experiment Design

Introduction

Anoectochilus roxburghii (Orchidaceae family) is a native plant in some southern regions of China, such as Fujian, Guangdong, Hainan, Guangxi, Yunnan, Guizhou and Sichuan. Its distribution is also found in Taiwan. In recent years, *Anoectochilus roxburghii* has become much more well-known because of its special medicinal and ornamental value. *Anoectochilus roxburghii* produced in China is usually divided into two types: North *Cordyceps sinensis* and South *Anoectochilus roxburghii* (Li, 2001; Zhong, 1997). Through a long history, the *Anoectochilus* folk is more widely known by its laudatory names in China, such as Golden Grass, King of Medicine and Magic pill. In the Southeast Asia including Fujian and Taiwan areas, it has been used as a rare and precious medicinal herb traditionally for hundreds of years. Due to its great effect on the treatment of many diseases, such as pulmonary tuberculosis, lung cough, rheumatoid arthritis, infantile convulsion and injuries caused by snakes and insects (see Wu, Chuang, Yang and Lin, 2010; Refish, Talib, Gu, Fu and Yu, 2016). The effect of *Anoectochilus* on the treatment of high blood pressure, diabetes and cancer has been proved recently, which makes it increasingly draw the attention from both researchers and professionals (Wu, Fen and He, 2008; Liu, Liu, Xiao, Zhou, Zhang and Li, 2013; Yu, Lian, Wu, Yu, Xie and Wu, 2015). But because of the over-collected and the loss of its suitable habitats, *Anoectochilus* is now in extinct condition (Zhang, Wang, Shao, Xua, Zhang and Li, 2015). The germination rate of the tiny seeds of *Anoectochilus* is very low and the less developed roots prevent the plants from growing fast in natural reproduction. Besides, the mountain areas are being developed fast for the economic benefits, the ecological conditions for the natural growth of *Anoectochilus*. Combined with the destruction problem caused by pest birds, wild *Anoectochilus* populations are declining sharply and on the verge of extinction (Wei, Xie, Qin and Chen, 2012; Xiao, Lai, Lin and Wu, 2014; Chen and Chen, 2014; Huang, 2015). Therefore, researchers in Fujian, Taiwan and Japan started conducting researches about its cultivation actively in recent years (Shao,

Zhou, Hu, Zhang, Liu and Li, 2014; Wei, Xie, Qin and Chen, 2015^a); Chen, 2015).

The artificial cultivation of *Anoectochilus roxburghii* is of crucial significance because it is the prerequisite for further research on its application for medicinal purposes (Zhang, 2004). This paper focuses on the issue of how to produce high-quality *Anoectochilus roxburghii* at relatively low cost.

Previous Study on Product Quality and *Anoectochilus*

Whenever the term product quality is discussed, price has often been used as an indicator for its measurement (Maynes, 1976). Deming (1982) defined quality as “a predictable degree of uniformity and dependability, at low cost and suited to the market.” “Differences in product quality reflect differences in the quantity of some ingredient or attribute possessed by a product” (Garvin, 1984). Therefore, Product quality tends to be threatened when there is an excessive control of cost in the production process, because the producers might have to replace the expensive ingredients with affordable materials (Leffler, 1982; Lancaster, 1971). The value of *Anoectochilus* is mainly embodied through its medicinal ingredients, namely, polysaccharide, flavones and alkaloids, which are significant indicators for its quality. How to achieve high-quality *Anoectochilus* at low cost to meet the increasing market demand is a challenge for advanced research in artificial cultivation.

There have been many attempts in artificial cultivation of *Anoectochilus*. Studies report progress in Japan (Akihisa, 2007), Pakistan (Ayub, 2008), India (Anilkumar, 2007) and Nepal (Pandey, 2006). The artificial cultivation techniques are believed to have been applied by more than 100 countries and areas in the world. Artificial cultivation techniques can greatly shorten the growth period of *Anoectochilus* and overcome the adverse effects on the plant itself caused by continuous planting on the same pieces of land. Culture solution and fertilizers are widely used to achieve high yield, good quality and less polluted *Anoectochilus* (Sasaki, 1998). Tissue culture technique has also been applied to the artificial cultivation of *Anoectochilus roxburghii* by many researchers (Chen, Lin, Zou and Lin, 2008; Piao, Chakarabarty, Hahn and Paek, 2003; Liu, Wei and Cen, 2009; Cen, Wei and Liu, 2009; Kan, Zhang and Li, 2009; Liu and Wang, 2009). Although the technique has been able to achieve some success, a decisive breakthrough is still to be expected. *Anoectochilus* has long been in short supply or even out of stock both at the domestic market in China and abroad (Zhao, 2008). Therefore, it is of crucial significance to conduct detailed investigation on the ecological habit and quality control of *Anoectochilus roxburghii* in order to discover effective methods to increase its amount of production and improve its quality as well.

Technical management level has reached an unprecedented increase in the scope of business activities, realized intensive technicalization and industrialization, and achieved the goal of good quality, high yield, high efficiency and low cost in regional production (Chen, Lin, Zou and Lin, 2008; Zhao and Guo, 2011; Wei, Xie, Qin and Chen, 2015^b). According to Wang and Hao (2003), three steps are needed for quality analysis before any quality-related problems can be solved: hazard analysis, cause analysis and problem solution analysis. Sound scientific methods, such as statistic techniques, should be employed to analyze the exact cause of the quality-related problems on a knowledge basis. Decision-making process should be established on the result of quantitative analysis and systematic testing (Jia, 2014). You, Zhou and Wu et al. (2014) further

explains in detailed the indispensable practice for quality analysis, such as pareto diagram, cause and effect diagram, histogram, variance analysis, signed rank sum test, hypothesis testing and orthogonal experimental design. Thus, with the help of such statistical analysis techniques, high-standard quality management can be achieved for the artificial cultivation of *Anoectochilus roxburghii*. The improvement of its output quality will expand its scope in both application and business.

This paper analyzes the artificial cultivation techniques of *Anoectochilus roxburghii* from the perspective of quality analysis and compares the *Anoectochilus* production process in Fujian and Taiwan. The purpose of this study is to put forward corresponding countermeasures and suggestions on how to boost the quality of the output.

Model Specification and Methodology

Rank Sum Test

The rank sum test is also commonly called the Mann-Whitney rank sum test or simply the Mann-Whitney test. It is “a nonparametric alternative to the two sample t-test which is based solely on the order in which the observations from the two samples fall” (Wild, 1997). When the populations are non-normal, particularly for small samples, then the t-test may not be valid as a standard test for testing that the difference between population means for two non-paired samples are equal. The rank sum test is an alternative that can be applied when distributional assumptions are suspect. It is one of the significant testing methods for data with uncertain population distribution.

In this study, *Anoectochilus* produced in Fujian and Taiwan are selected as samples and the differences between these two varieties are to be discovered. Because the distribution of collected data is not known, rank sum test is used for data analysis.

Orthogonal Experimental Design Method

Orthogonal design is “a powerful method for comparative effectiveness research with multiple interventions” (Zurovac and Brown, 2012) and has been widely applied to research within the management and manufacturing fields. It is a quantitative analysis method which can find out the influence degree of each individual factor on the problem, so as to accurately determine the weak factors among a whole and provides reliable evidence for further quality analysis. In this study, orthogonal experimental design method is used to discover factors causing problems for the artificial cultivation of *Anoectochilus roxburghii* and offer suggestions on the improvement of its quality management.

Comparative Analysis of *Anoectochilus roxburghii* using Rank Sume

Test

There are strict environmental regulations for the artificial cultivation of high-quality *Anoectochilus roxburghii*. The temperature requirement is $15^{\circ}\text{C} - 30^{\circ}\text{C}$, and humidity should be controlled between 60% and 80%. Generally speaking, light intensity should be adjusted within the range of 3000 – 3500 lx. The experiment was conducted with the support of L. Company located in Zhejiang, China. Its Greenhouse Cultivation Lab provides an ideal environment for the cultivation of *Anoectochilus roxburghii*. The temperature, humidity and light intensity was kept at a most favorable level as described above with utmost care and attention so that the plant was able to achieve and sustain desirable growth. Two culture disks were set up in the lab, with one labeled as Fujian *Anoectochilus roxburghii* and the other labeled as Taiwan *Anoectochilus roxburghii*. There are 200 plants in each of the disks. The whole cultivation process lasts for 90 days. The end products are collected after the cultivation period. In order to collect samples for this study with no discrimination, ten plants were randomly selected from each disk and weighed using a special electronic scale after being washed and dried. The net weight data of the collected *Anoectochilus roxburghii* are obtained as in table 1.

Table 1 Net Weight of *Anoectochilus roxburghii*

Districts		Net weight (10 mg)									
Fujian	92	78	76	87	89	94	90	88	85	88	
Taiwan	65	69	86	80	68	63	76	82	75	66	

The rank sum test for the data in table 1 is conducted. The rank table (Table 2) is achieved according to the data size order. A stands for Fujian *Anoectochilus roxburghii*, and B stands for Taiwan *Anoectochilus roxburghii*.

In this study, $n_A = n_b = 10$ is a prerequisite for the rank sum test. The sum of ranks (T_A) of group A is $T_A = 7.5 + 9 + 12 + 14 + 15.5 \times 2 + 17 + 18 + 19 + 20 = 147.5$. The sum of ranks (T_B) of group B data is $T_B = 1 + 2 + 3 + 4 + 5 + 6 + 7.5 + 10 + 11 + 13 = 61.5$. The statistic is $Z = \min(A, B) = 61.5$.

Given that the significant level of α is 0.05, $T_A = 83$ and $T_B = 127$ can be obtained through checking the rank sum test table. Therefore, it is included that if $T_A \leq T \leq T_B$, the null hypothesis H_0 is acceptable (There is no significant difference between A and B) is accepted; if $T < T_A$ or $T > T_B$, the null hypothesis H_0 is rejected (There is a significant difference between the quality of *Anoectochilus roxburghii* produced in Fujian and Taiwan despite the same cultivation conditions. The quality of Fujian *Anoectochilus roxburghii* is better than that of Taiwan).

Table 2 Net Weight Order of *Anoectochilus roxburghii*

ranks		A	B
	1		63
	2		65
	3		66
	4		68
	5		69
	6		75
7.5	7		76
	8	76	
	9	78	
	10		80
	11		82
	12	85	
	13		86
	14	87	
15.5	15	88	
	16	88	
	17	89	
	18	90	
	19	92	
	20	94	

Improvement of The Output Quality of Fujian *Anoectochilus*

Roxburghii

The valuable ingredients of *Anoectochilus roxburghii* for medicinal use are polysaccharide, flavones and alkaloids. The result of the rank sum test suggests that more effective ingredients are contained in Fujian *Anoectochilus roxburghii* than Taiwan *Anoectochilus roxburghii*. In order to promote the quality of Fujian *Anoectochilus roxburghii* and obtain the strongest seeding for future artificial cultivation, an experiment is conducted in this study by mixing up humus soil, river sand and perlite with three mediums in different amounts - MS, AR and SE. The purpose of this is to create a most fertile and productive soil for the cultivation of *Anoectochilus roxburghii* and confirm the most favorable conditions for cultivation so that the effective ingredients of the produced *Anoectochilus roxburghii* could reach the maximum level. The analysis process consists of the following three steps:

- Step 1: Set up a clear objective for the test and determine evaluation index.

As explained above, the objective is to discover the most suitable combination of the environmental factors, which affects the growth of *Anoectochilus roxburghii* and enhance the quality of the output. Therefore, the most significant evaluation index is the amount of effective ingredients included in the final produced *Anoectochilus roxburghii*.

- Step 2: Select factors for the different levels.

Four factors and three levels are identified according to the theory and practice for the experiment as in Table 3.

Table 3 Factor Level Table

Levels	Factor			
	Mediums	Humus soil	River sand	Perlite
1	MS	400	300	300
2	AR	500	300	200
3	SE	600	200	200

- Step 3: Create the appropriate orthogonal table.

Four factors and three levels from the directions for the orthogonal table $L_9 (3^4)$. Fujian *Anoectochilus roxburghii* was cultivated for a 90-day grows cycle in a most desirable cultivation environment as described above. After the cultivation, the collected sample plants were sent to an authoritative inspection and quarantine organization for measurement of the three crucial ingredients - flavonoids, alkaloids and polysaccharide. Table 4 shows the detailed data.

Table 4 Ingredients of Fujian *Anoectochilus roxburghii*

Levels	Mediums	A	B	C	D	E	F	Total(D+E+F)
1	MS	400	300	300	41	38	25	104
2	MS	500	300	200	42	32	27	101
3	MS	600	200	200	53	39	28	120
4	AR	400	300	300	38	29	22	89
5	AR	500	300	200	31	35	17	83
6	AR	600	200	200	39	30	25	94
7	SE	400	300	300	41	26	18	85
8	SE	500	300	200	31	30	29	90
9	SE	600	200	200	45	25	22	92

Note: A: Humus soil, B: River sand, C: Perlite, D: Polysaccharide, E: Flavones, F: Alkaloids

Table 5 Homogeneity Test of Variances

		N		
Medium	AR	9		
	MS	9		
	SE	9		
Additive	A	9		
	B	9		
	C	9		
<hr/>				
F	df ₁	df ₂	Sig.	
1.611	8	18	0.190	

According to the evaluation criteria of L. Company, the three important ingredients in *Anoectochilus roxburghii*, polysaccharide, flavonoid and alkaloid, should not go below $50 \pm 3\text{mg}$ and $30 \pm 3\text{mg}$, respectively. The higher the amount for each ingredient, the better of the quality. That is, *Anoectochilus roxburghii* with the highest amount of polysaccharide, flavonoid and alkaloid is considered the top level in the area of quality. Through comparison of the total amount of effective ingredients in table 4, conclusion is made that the best-quality Fujian *Anoectochilus roxburghii* is available by employing the medium (MS) with humus soil(600), river sand (200) and perlite (200).

- Step 4: Verify the result

SPSS 16 software is used to verify the result through homogeneity test of variances. The output is shown as in table 5.

The homogeneity test of variances for univariate analysis is indicated as in table 6. According to table 6, the sig. value is 0.190, more than the significant level 0.05. This shows that the data meets the homogeneity of variance assumption. The effect test results are shown in table 6.

The corrected Model in Table 6 is the test of the variance analysis model. All factors in the model including the medium and the additive (A, B, C) have no significant effects on the cultivation output of Fujian *Anoectochilus roxburghii*. The value of significant level for the Corrected Model is close to zero, which implies that the proposed model is a significant fit for the data. The Intercept in Table 6 is a constant item in the model. It has no practical significance in the analysis and should not be taken into account. The significance levels of the Medium and the Addictive items in Table 6 are indicative because both sig. values are less than 0.05 (α). This means that both the medium and the additive (A, B and C) have a significant effect on the quality of the cultivated *Anoectochilus roxburghii*. The sig. value of the fifth item (Medium * Additive) is 0.624, which is much greater than 0.05 (α).

This implies that the interaction of medium and additive is not significant in this study. The multiple decision coefficient $R - Squared$ is 0.795. This shows that the factor medium explains 79.5%

Table 6 Effect Test Results

Sources	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	159.333	8	191.167	8.748	0.000
Intercept	27 265.333	1	27 265.333	1.248E3	0.000
Medium	253.556	2	126.778	5.802	0.001
Additive	1 217.556	2	608.778	27.859	0.000
Medium * Additive	58.222	4	14.556	0.666	0.624
Error	393.333	18	21.852		
Total	29 188.000	27			
Corrected Total	1 922.667	26			

$R - Squared = 0.795$ ($AdjustedR - Squared = 0.704$)

Table 7 Multiple Comparison of Medium and Addictive

(I)	(J)	Mean Difference (I-J)	Std.Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	8.555 6*	2.203 63	0.001	3.925 9	13.185 2
	C	16.444 4*	2.203 63	0.000	11.814 8	21.074 1
B	A	-8.555 6*	2.203 63	0.001	-13.185 2	-3.925 9
	C	7.888 9*	2.203 63	0.002	3.259 2	12.518 5
C	A	-16.444 4*	2.203 63	0.000	-21.074 1	-11.814 8
	B	-7.888 9*	2.203 63	0.002	-12.518 5	-3.259 2

*The mean difference is significant at the 0.05 level.

Table 8 Estimates of the Marginal

Mean	Std.Error	95% Confidence Interval	
		Lower Bound	Upper Bound
31.778	0.900	29.888	33.668

relies on other random factors.

Multiple comparison results of these two items, medium and additive, are presented in Table 7. Table 8 shows the marginal estimates of the quality of Fujian Anoectochilus roxburghii.

Conclusions

In order to find out the most productive environment for the cultivation of *Anoectochilus roxburghii*, this study carries out a comparative study to investigate the quality of Fujian *Anoectochilus roxburghii* and Taiwan *Anoectochilus roxburghii*, which were cultivated under the same conditions, with controlled temperature, humidity and light intensity. The rank sum test shows that there is significant difference between these two items in term of output quality. Fujian *Anoectochilus roxburghii* has more effective ingredients than the Taiwan product. This establishes the foundation for further study on how to improve the quality of the Fujian *Anoectochilus roxburghii*. Different combinations of mediums and additives are employed for the cultivation and the quality of *Anoectochilus roxburghii* cultivated under different condition is investigated. The combination of medium MS, humus (600), river sand (200), perlite (200) proves to be the constituents for the best growth environment of *Anoectochilus roxburghii* based on data of repetitive experiments.

The statistical analysis achieves good results with a sound argument. However, there are limitations in this study due to the relatively small size of samples. More efforts are needed to modify the cultivation conditions for high-quality *Anoectochilus* growth. More practical data is needed to validate the effectiveness of the mixture of medium AR and SE with different additives - humus soil, river sand and perlite for the cultivation of *Anoectochilus roxburghii*. The potential effects on the interaction of three mediums (MS, AR and SE) and additives (humus soil, river sand and perlite) are to be further explored by using more sample data. In *Anoectochilus roxburghii*. Both the methods and the construction put forward in this study will lead to the construction of a through framework of practice for the cultivation of high-quality *Anoectochilus roxburghii*.

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