Separating and Purifying of Endophytic Fungi from Ginkgo Biloba and Screening of Flavonoid-Producing Strains

X.Y. Zhang1,2, X. Li1,2, M.M. Han1,2, Z.Y. Cai1,2, X. Gao1,2, M.X. Pang1,2,3,4, J.H. Qi1,2,3,4, F. Wang1,2,3,4,*

1Beijing Key Laboratory of Agricultural, Product Detection and Control of Spoilage Organisms and Pesticide Residue, Beijing University of Agriculture, Beijing 102206, China.
2The Teaching Group of Food Chemistry, Faculty of Food Science and Engineering, Beijing University of Agriculture, Beijing 102206, China.
3Beijing Innovation Consortium of Swine Research System, Beijing 102202, China.
4Graduation Design of the “Practical Training Program” for the Cross-Cultivation of High-Level Talents in Beijing Colleges and Universities, 2018.
*Corresponding author’s e-mail: 254221029@qq.com

Abstract: In order to screen the endophytic fungi of ginkgo biloba with high flavonoid yield, the surface of the sample was disinfected by immersing with 75% ethanol, 3% sodium hypochlorite and sterile water alternating. The endophytic fungi of ginkgo biloba were separated by tissue culture method. 47 strains of endophytic fungi were obtained, and 16, 17 and 14 strains were found in the branches, leaves and roots respectively. The flavonoid-producing strains and its content of total flavonoids in the fermentation broth were determined by chromogenic reaction and colorimetric method of aluminum chloride respectively. Finally, four strains with the highest flavonoid-producing (Y6, Y8, Y10 and G8) were screened, and theirs total flavonoids content in the fermentation broth were 25.33±1.29 mg/L, 32.04±1.38 mg/L, 20.48±0.83 mg/L and 20.19±1.59 mg/L respectively.

1. Introduction
Ginkgo flavones have a variety of biological activities. According to a large number of research results at home and abroad, ginkgo flavones have significant pharmacological effects on antioxidant, anti-tumor, and improvement of cardiovascular and cerebrovascular diseases, and some of them have been made into drugs for clinical treatment[1-3]. However, since the shortage of ginkgo resources and the limitation of season and region, the preparation of ginkgo flavone is faced with many problems. Endophytes were defined as microorganisms that lived in plant tissues but do not cause diseases during a certain period of plant life[4]. Abundant secondary metabolites could be produced by some endophytic fungi. In 1993, an endophytic fungus was isolated from the phloem tissue of yew by Strobel et al, and its metabolites contained taxol a new anticancer substance[5]. The inextricable internal connection within endophytic fungi and plants was realized by people gradually. In this paper, the endophytic fungi of ginkgo were isolated and purified, then strains screened that could provide flavonoid so as to offering a new research direction for the problem of ginkgo flavones.
2. Materials and methods

2.1 Collect and process of sample
In October 2017, a healthy and disease-free ginkgo tree was selected in Beijing University of Agriculture, Chang Ping district, Beijing, and three parts of its roots, branches and leaves were collected, packaged and labeled in sterile sealed bags, then stored under 4 °C. The isolation test of endophytic fungi was completed within 24 h.

2.2 Surface disinfection of plant tissue
The surface of plant tissue was cleaned with detergent, and dry it for later. Do not scratch the surface during cleaning. The disinfection method is shown in the following table:

| plant tissue | Disinfection procedures | 75% ethanol immersion | Sterile water rinse | 3% NaClO Sterile water rinse | 75% ethanol immersion | Sterile water rinse |
|--------------|-------------------------|-----------------------|--------------------|----------------------------|-----------------------|--------------------|
| leaves       | 1min                    | 3 times               | 1min               | 3 times                    | 30s                   | 3 times            |
| branches     | 1min                    | 3 times               | 3min               | 3 times                    | 30s                   | 3 times            |
| roots        | 1.5min                  | 3 times               | 5min               | 3 times                    | 40s                   | 3 times            |

2.3 Separating and Purifying of endophytic fungi from ginkgo biloba
Tissue separation and homogenization smear both included in separation methods of endophytic fungi[6,7]. In this paper, the endophytic fungi would be isolated by tissue separation. Ginkgo leaves were cut into 0.5 cm × 0.5 cm chunks. The branches and roots were cut into 0.5 cm long and cut from the middle vertically. Every four identical tissues were implanted in one PDA petri dish. Sterile water from the last rinsing of plant tissues was coated on the surface of culture medium for blank control, for observing whether the surface of plant tissues was sterilized completely.

The petri dish was placed in 28 °C cultured for 5-7 days. When mycelia or colony was generated on the edge of tissue, it was isolated by tip pick method and separated for several times until there was a single colony in the petri dish.

2.4 Named method of strains
The strains were named with the combination of capital letters and Arabic numerals. The roots, branches and leaves were represented by G, Z and Y respectively. The strains from the same parts were distinguished by Arabic numerals, such as G1, Z5, Y12, etc.

2.5 Methods for preserving strains
Low-temperature inclined plane and aseptic water short-term preservation and glycerol long-term preservation were included in the preservation methods of fungi. Low-temperature inclined plane and glycerol methods were adopted in this paper. The storage periods of them are 3-6 months and more than 2 years, respectively.

2.6 The Preparation of samples for chromogenic reaction
The endophytic fungi separated were vaccinated to PDA medium, then picked 0.5 cm × 0.5 cm to conical flask (250 ml) with 100 ml PD medium after culturing at 28 °C for 5-7 days. The fungal fermented filtrates would be received after culturing at 28 °C, 150 r/min shaking for 7 days. Each strain was cultured three bottles. In order to destruct fungal cells and dissolving intracellular material, the fungal fermented filtrates were processed ultrasonic in 30 °C, 45 kHZ for 30 min. The fungal fermented filtrates were extracted with ethyl acetate and n-butanol for three times in a ratio of 5:2 (50 mL fermentation liquid, 20 mL extraction agent) respectively after removed Mycelia. The extraction liquid was dried over
Rotary Evaporator under 50 °C, then redissolved in 4 mL methanol solution and stored in 4 °C refrigerator after 0.45 m filtration membrane for testing.

2.7 Chromogenic reaction
The following chromogenic reactions are adopted in this paper[8-10]:

(1) NaOH: An appropriate amount of 4% NaOH solution was dropped to 1 mL of the sample to be tested. If the color of solution changed form yellow to brown, that means the sample may contain flavonoids.

(2) FeCl₃: An appropriate amount of 2% FeCl₃ solution was dropped to 1 mL of the sample to be tested. If the color of solution changed to wine red, that means phenolic hydroxyl group might include in the sample.

(3) Concentrated sulfuric acid (85%): An appropriate amount of concentrated sulfuric acid was dropped to 1 mL of the sample to be tested. The samples were determined according to Table2.

Table2. The color reaction of sulfuric acid (85%)

| flavone     | flavonol  | flavanone | chalcone | isoflavone | aurone  |
|-------------|-----------|-----------|----------|------------|---------|
| yellow→orange | yellow→orange | orange→purple | Orange, purple | yellow red, magenta |

2.8 Determining of total flavonoids
Aluminium chloride colorimetry was used to determining the content of total flavonoids in the fungal fermented filtrates[11]. 1% of AlCl₃ solution was added to 1 mL sample until volume to 5 mL, then shaken well and let stand for 10 min to test by ultraviolet spectrophotometer at 420 nm wavelength, with 1% of AlCl₃ solution as CK. The total amount of flavonoids in the sample was calculated according to the amount equivalent to 320 µg of flavonoids when A=1. The formula is as follows:

$$\text{Total flavonoids (µg)} = \frac{A \times 320 \times V_T}{V}$$

(1)

Vₜ : The total volume of the sample to be tested,  V: The sample volume.

3. Result and analysis

Figure1. Number of endophytic fungi isolated from different parts of ginkgo

The figure1 shows that different tissues of ginkgo biloba all could be isolated endophytic fungi. The number of endophytic fungi isolated form leaves, branches, roots were 16, 14, 17 respectively, in total 47 strains. The different number of endophytic fungi isolated form different various parts of the plant may be related to the survival environment of the different parts of the organization. The moisture content of ginkgo biloba leaves is higher than branches and roots. The complex bacteria in the soil structure may affect the endophytic fungi population.
Table 3. The result of chromogenic reaction

| Strains | 1  | 2  | 3  | Strains | 1  | 2  | 3  | Strains | 1  | 2  | 3  |
|---------|----|----|----|---------|----|----|----|---------|----|----|----|
| Y1      | +  | +  | +  | Y13     | +  | +  | -  | Z8      | +  | +  | +  |
| Y2      | +  | +  | +  | Y14     | -  | -  | +  | Z9      | +  | -  | -  |
| Y3      | +  | -  | -  | Y15     | -  | +  | +  | Z10     | +  | +  | +  |
| Y4      | +  | -  | -  | Y16     | -  | +  | -  | G6      | -  | +  | +  |
| Y5      | +  | -  | -  | Y17     | -  | +  | +  | G8      | +  | +  | +  |
| Y6      | -  | +  | -  | Z1      | +  | +  | -  | G9      | -  | +  | +  |
| Y7      | -  | -  | -  | Z2      | +  | -  | -  | G10     | +  | +  | +  |
| Y8      | +  | +  | +  | Z3      | -  | +  | -  | G11     | +  | -  | +  |
| Y9      | +  | -  | -  | Z4      | +  | -  | -  | G12     | +  | +  | -  |
| Y10     | +  | -  | -  | Z5      | -  | +  | +  | G13     | -  | +  | +  |
| Y11     | +  | -  | -  | Z6      | -  | +  | -  | G14     | -  | +  | +  |
| Y12     | -  | -  | -  | Z7      | +  | -  | -  | G15     | +  | +  | +  |

*“+” in the table means positive reaction, “-” means negative reaction.

"1" means NaOH chromogenic reaction, “2” means AlCl₃ chromogenic reaction, “3” means concentrated sulfuric acid chromogenic reaction.

The total flavonoid content

The molecular structure of flavonoids is derivated by 2-phenyl chromogen as parent nucleus among the structure the phenol hydroxyl and carbonyl ketone were contained. It could be identified by chromogenic reaction[12]. It could be seen from table 2 that there were much fungal metabolites of strains showed positive reaction with NaOH, FeCl₃ and H₂SO₄ (85%), and there are 13 strains were all positive results, which could be proved that the flavonoids were existence in those strains fermentation liquor. At the same time, it also indicates that endophytic fungi and ginkgo biloba coevolved to produce substances with the same or similar chemical properties, as a result of long-term parasitize in ginkgo structure and the influences of internal environment of plant and genetic.

Figure 2. Flavonoids content of the strains
It can be seen from the figure that the total flavonoids content of 6 strains in the middle part of the 13 endophytic strains are the highest. 4 strains with the highest flavonoids after comparing namely Y6, Y8, Y10 and G8, were screened out, and their total flavonoids content was 25.33±1.29 mg/L, 32.04±1.38 mg/L, 20.48±0.83 mg/L and 20.19±1.59 mg/L, respectively.

4. Conclusion
Sixteen, seventeen and fourteen endophytic fungi were isolated from the branches, leaves and roots of ginkgo biloba by tissue culture respectively. The fermentation broth of 47 strains was characterized by NaOH, AlCl₃ and concentrated sulfuric acid colorimetry. Aluminium chloride colorimetry was used to determine the total flavonoids content of 13 endophyte strains, and finally four endophyte strains with the highest flavonoids content were obtained, with the total flavonoids content of 25.33±1.29 mg/L, 32.04±1.38 mg/L, 20.48±0.83 mg/L and 20.19±1.59 mg/L, respectively.

References
[1] Liebgott T, Miollan M, Berchadsky Y, et al. (2000) Complementary cardioprotective effects of flavonoid metabolites and terpenoid constituents of Ginkgo biloba extract (EGb 761) during ischemia and reperfusion. J. Basic Res Cardiol. 95: 368 ~ 77.
[2] Diamond BJ, Shiflett SC, Feiwel N, et al. (2000) Ginkgo biloba extract: mechanisms and clinical indications. J. Arch Phys Med Rehabil. 81: 668 ~ 78.
[3] De Feudis FV, Drieu K. (2000) Ginkgo biloba extract (EGb 761) and CNS functions: basic studies and clinical applications. J. Curr Drug Targets. 1: 25 ~ 58.
[4] Orlando P. (1991) Fungal Endophytes of Tree Leaves. J. Microbial Ecology of Leaves, 179 ~ 197.
[5] Strobel G, Stierle A, Stierle D. (1993) Taxomyces andreana a proposed new taxon for a bulbiliferous by phomycete associated with pacific yew (taxus brevifolia). J. Mycotoxon, 40: 71 ~ 81.
[6] Vendan R T, Yu Y J, Lee S HS, et, al. (2010) Diversity of endophytic bacteria in ginseng and their potential for plant growth promotion. J. The Journal of Microbiology, 48: 559 ~ 565.
[7] He, J., Liu, X.J., Zhao, Q.M., Chen, J. (2009) Isolation of endophytes from pseudolarix kaempferi gord. J. Food Science, 30: 180 ~ 183.
[8] Tang, H.G, et, al. (2009) Studies on flavonoids. Science Press, Beijing.
[9] Wueren, A., Guo, Y.F, et, al. (2017) Screening and identifying of flavonoids endophytic fungi from sauschia tianshan. J. Chinese Traditional Patent Medicine, 39: 2424 ~ 2427.
[10] Wang, J.Z., Lv. H.C. (2013) Content Determination of total flavonoids in resina draconis by colorimetric method. J. Chinese Journal of Experimental Traditional Medical Formulae, 19: 55-58.
[11] He, Z.F., Zhang, D.Q. (1998) Health food chemistry and its detection technology. China Light Industry Press, Beijing.
[12] Danny E.C Van Hoorn, Robert J Nijveldt, Paul A.M Van Leeuwen, et al. (2002) Accurate prediction of xanthine oxidase inhibition based on the structure of flavonoids. J. European Journal of Pharmacology, 451: 111 ~ 118.