Isolation and identification of endophytic fungi in walnut

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Abstract. In this paper, endophytic fungi in four tissues (roots, branches, leaves, fruits) of walnuts were isolated by tissue isolation technic. Based on morphology, the isolated endophytic fungi were preliminarily identified. The results showed that 64 strains of endophytic fungi were isolated from walnut tissues. The number of endophytic fungi isolated from walnut branch tissues is the largest, followed by leaf, fruit, and root tissues. Observing the morphological and mycelial changes during fungal growth and the characteristics of spore morphology under the microscope, 64 strains of endophytic fungi belonged to 17 genera, of which Alternaria sp. was the dominant microflora. For the first time, Talaromyces sp., Curvularia sp. and Eurotium sp. were isolated from walnut plants.

1. Introduction
Endophytic fungi inhabit widely inside different tissues of the host plant, without inducing any apparent expression of any disease (Gupta et al., 2020). During long-term coevolution with host plants, endophytic fungi and host plants form a complex interaction mechanism (Zhao et al., 2016; Nathan et al., 2020). It was found that the flora structure of endophytic fungi was affected by plant species, parts, growth stage and growth environment, showing a certain preference (Hakimeh et al., 2019; Zheng et al., 2013). Some endophytic fungi could promote the accumulation of plant active components, and some could produce components similar to the host structure or function through secondary metabolism. Zhang et al. isolated and screened an endophytic fungus named G10 from forsythia, which could produce forsythia glycoside, an effective component of forsythia (Zhang et al., 2012). Shan et al. screened endophytic fungi with strong antibacterial and antioxidant activities from Casuarina equisetifolia (Shan et al., 2019). The secondary metabolites produced by endophytic fungi not only can be used as substitutes for the functional components of plants, but also provide another solution for our production of functional components. Endophytic fungi become a new microbial resource with great potential.

With economic and medicinal value, walnut is one of the most important cultivated tree species in the world. Green husk, leaves, branches, flowers and shells of walnut all can be used as medicine. The extracts from various tissues of walnut had superior performance in antioxidant and bacteriostatic abilities (Wei et al., 2018, 2019; He et al., 2019). In recent years, with the development of walnut research, abundant endophytic fungi are found in walnut, which have great potential for bioactivity...
development. Recent research by Hui screened endophytic fungi from walnut and studied their antibacterial mechanism, and the results showed that the fermentation products of G3, from walnut root, showed certain antibacterial activity against 6 pathogens (Hui, 2019). In Pang et al.’s study, the strain lst-6-6 (Pyrenochaeta) with high antioxidant activity of metabolites were screened, which indicated that endophytic fungi of walnut were a good resource for searching for natural antioxidant active substances (Pang et al., 2019).

At present, the influence mechanism of plants on endophytic fungi is not clear, and the research on the diversity of endophytic fungi in walnut is less. The purpose of this study is to isolate and purify endophytic fungi from walnut tissue, and to identify the strains by morphology, so as to lay the experimental foundation for further diversity research and the development and utilization of endophytic bacteria.

2. Materials and methods

2.1. Materials and regents
Randomly selected three healthy and disease-free walnut trees, planted in Institute of Forestry and Pomology, affiliated to Beijing Academy of Agricultural and Forestry Sciences. The tissues collected from roots, branches, leaves, and fruits of these trees, were stored in sterile sealed bags.

Glucose and agar were purchased from Sinopharm Chemical Reagent Co., Ltd. Chloramphenicol was received from Beijing Changhua Zhicheng Technology Co., Ltd. Hypochlorous acid (analytical purity), absolute ethanol (analytical purity) were purchased from Beijing Chemical.

2.2. Preparation of culture medium
Potato-dextrose agar (PDA) medium was prepared by traditional methods. Typically, 200 g of fresh potatoes were cut into small cubes and followed by the addition of appropriate amount of water and kept in a boiled state for 30 min. After passing through 8 layers of gauze, the residue in it was removed, and then 20 g of glucose, 15 g of agar and 30 mg of chloramphenicol were added to dissolve, autoclaved at 121 ℃ for 20 min (YXQ-LS-50, Shanghai Boxun Industrial Co., Ltd.) and poured into a sterile petri dish for use.

2.3. Disinfection of plant tissue surface
The surface of the plant tissues in the sterile bag was washed with sterile water and dried for use. The solution of ethanol and sodium hypochlorite common disinfection was adopted to ensure the complete sterilization of the plant tissue surface. The disinfection method was based on the method described by Zhang with some modifications (Zhang, 2014). The disinfection method was shown in Table 1.

| Sampling position | 75% ethanol immersion (minutes) | Sterile water rinse (times) | 3% sodium hypochlorite soak (minutes) | Sterile water rinse (times) | 75% ethanol immersion (seconds) | Sterile water rinse (times) |
|-------------------|-------------------------------|--------------------------|--------------------------------------|--------------------------|-------------------------------|--------------------------|
| Roots             | 1                             | 3                        | 1                                    | 3                        | 30                            | 3                        |
| Branches          | 1                             | 3                        | 3                                    | 3                        | 30                            | 3                        |
| Leaves            | 1.5                           | 3                        | 5                                    | 3                        | 40                            | 3                        |
| Fruits            | 1                             | 3                        | 3                                    | 3                        | 30                            | 3                        |

2.4. Isolation and Purification of Walnut Endophytic Fungi
The separation methods of plant endophytic fungi included tissue isolation and homogenous smear method (Vendan, 2010; He et al., 2009). In this paper, tissue isolation was used to isolate endophytic fungi of walnut tissues. The method of inoculation was performed according to the procedure described by He et al (He et al., 2009). Before inoculation, the walnut leaves were cut into 0.5 cm ×
0.5 cm blocks. The walnut branches and roots were cut into 0.5 cm segments and cut vertically from the vertical. All the treated tissues were implanted into PDA Petri dishes separately, which can contain 4-5 similar tissues. The last sterile water used to rinse the plant tissue was applied to the surface of the culture medium as a blank control to observe whether the surface of the plant tissue was completely disinfected.

The petri dishes implanted into the tissues were placed in a 28 ℃ constant-temperature incubator (WS-01, HUANG SHI HENGFENG MEDICAL INSTRUMENTCO.LTD) and cultured for 5-7 days. When hyphae or colonies were formed at the edge of the tissue, the colonies were separated multiple times by the tip picking method until a single colony was in the petri dish.

2.5. Nomenclature of strains
The nomenclature of the isolated strains was designed with capital letters, lowercase letters, and Arabic numeral combinations. Strains isolated from different parts (branch, leaf, root, fruit) were represented by "Z", "Y", "Ge", and "G" respectively. Different strains in the same part were distinguished by Arabic numerals, such as "Z-10", "G-1", "Y-4" and "Ge-7".

2.6. The method of strain preservation
In this paper, periodic transplantation and glycerol-preservation were used to preserve the strains of walnut endophytic fungi. The periodic transplantation preservation was to inoculate fresh hyphae on the PDA slope culture medium cultivating at 28 ℃ for 3-5 days. After the colonies were ensured that there were no mixed bacteria, they were kept in a refrigerator (YC-300L, Zhongke Meiling Cryogenic Technology Co., Ltd.) at 4 ℃. The periodic transplantation preservation was simple and convenient, and the fungi could be stored for about 3-6 months. The long-term storage method was to mix the fermentation broth and glycerin for 5-7 days at a 1: 1 ratio, or put fresh mycelium in sterile 30% glycerin and store it at -80 ℃ for long-term storage.

2.7. Preliminary identification of strain morphology
The purified fungal hyphae were picked and inoculated on PDA plates, cultured at 28 ℃ for 5-7 days. During the days, the changes in colony morphology and hyphae colour were observed. The structure of fungal hyphae was observed with a microscope (ZEISS Axioplan 2, Carl Zeiss AG) with the water immersion method (Zhang et al., 2016). Observed the hyphae morphology, spore characteristics, and other characteristics (rhizoid, podocyte, stolon hypha, etc.) under the microscope. Refer to the manual of endophytic fungi identification to identify strains based on morphology (Wei, 1979).

3. Results and discussion

3.1. Isolation of walnut endophytic fungi
After multiple isolation and purification of the colonies, 64 strains of walnut endophytic fungi were finally obtained (Figure 1). According to the number of strains isolated, the number of endophytic fungi in the branch tissue was the largest, followed by leaf, fruit, and root tissue.

The various numbers of endophytic fungi isolated from different parts may be related to their living conditions. The water content of walnut leaves was higher than that of branches and roots, and walnut fruit contained a lot of juglone. Secondly, the complex microflora in the soil may also affect the formation of endophytic fungal populations.

In order to ensure that the endophytic fungi isolated from walnuts were free of mixed bacteria, the surface of plant tissues should be sterilized. At present, ethanol, sodium hypochlorite and mercury bichloride are the most widely used as disinfectants in China. However, ethanol disinfection is not complete, and it needs to be used together with other disinfectants; although sodium hypochlorite has a good disinfection effect, it has strong permeability and is not suitable for long-term immersion. Mercury as a result of its strong toxicity was easy to remain on the surface (Wang and Liu, 2014). The method of 75% ethanol and 3% sodium hypochlorite alternate disinfection was used in this study.
Although it had good sterilization effects, the microbial flora on the plant surface was complex and cannot guarantee complete disinfection. Secondly, in the process of soaking, sodium hypochlorite might penetrate into the tissues and destroy the endophyte population.

After conducting preliminary experiments, the widely used PDA medium is currently selected as the medium for endophytic fungi, but individual fungi may not be isolated due to the different nutrient requirements of individual fungi. The accuracy of the endophytic fungal population would be affected by the above factors.

Figure 1 The number of endophytic fungi isolated from different parts of *Juglone regia* L.

### 3.2. Preliminary identification of strain morphology

Observed the mycelium structure of the fungi, the characteristics of the spores and the changes of the colony morphology and mycelium colour during the cultivation process. With reference to the fungal identification manual, 64 strains of walnut endophytic fungi were determined to belong to 17 genera: *Alternaria* sp., *Fusarium* sp., *Neonectria* sp., *Phomopsis* sp., *Phoma* sp., *Diaporthe* sp., *Chaetomium* sp., *Coprinellus* sp., *Talaromyces* sp., *Curvularia* sp., *Eurotium* sp., *Penicillium* sp., *Ozonium* sp., *Flower Anthina* sp., *Ectostroma* sp., *Dothiorella* sp., *Sordariomycetes* sp. Among the 17 genera, *Alternaria* sp. was the dominant genus of endophytic fungi, accounting for 22% of the total endophytic fungi (Figure 2).

Figure 2 The components of endophytic fungi in *Juglone regia* L.

The endophytic fungal populations of different parts of the same walnut tree were preliminarily identified, and the dominant microflora was *Alternaria* sp, which was basically consistent with previous reports (Wang et al., 2008; Liu, 2009). In addition, *Eurotium* sp., *Talaromyces* sp., and *Curvularia* sp. were isolated for the first time in walnut tissue. It was shown that the endophytic fungi population of walnut was affected by the growth environment, growth years and different parts of the tree.
plant. This may be related to the plant's own nutrients, moisture content, environmental temperature and other factors. Further in-depth exploration and research are still needed.

3.3. **Morphological characteristics of endophytic fungi in walnut**

![Colony morphology of Alternaria sp., Diaporthe sp., Chaetomium sp.](image1)

![Micro-morphology of Alternaria sp., Diaporthe sp., Chaetomium sp.](image2)

4. **Conclusions**

In this study, using tissue isolation technics, 64 strains of endophytic fungi were isolated from walnut tissues. The number of endophytic fungi isolated from walnut branches was the largest, with leaf, fruit and root tissues following. By observing the morphology and hypha changes during fungal growth, and the characteristics of spore morphology under the microscope, referring to the manual of endophytic fungi, 64 strains of endophytic fungi were identified as belonging to 17 genera, including Alternaria sp. as the predominant flora. In addition, Talaromyces sp., Curvularia sp. and Eurotium sp. were isolated from walnut plants for the first time.

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