Full Length Article

Occurrence of Root Rot of *Panax notoginseng* Caused by *Fusarium oxysporum* in China

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Abstract

A root rot disease was found in *P. notoginseng* plantations based in Guizhou province in 2015 and 2016. This disease affected mainly *P. notoginseng* rhizomes and leaves, causing oval or irregular spots, finally resulting in the infected plants withering and dying. We collected twenty-five infected rhizome samples of *P. notoginseng* from Guizhou province. The pathogen was identified as *Fusarium oxysporum* based on the morphology and molecular characterization including rDNA ITS, and EF sequence analysis, High throughput sequencing and Maximum Parsimony of the pathogen was conducted. In addition, we investigated the occurrence and frequency of *P. notoginseng* root rot in Guizhou province, and found that temperature and humidity were the main factors influencing *P. notoginseng* root rot. In addition, high light levels and lack of crop rotation were also associated with the occurrence of *P. notoginseng* root rot. © 2018 Friends Science Publishers

Keywords: *Panax notoginseng*; Root rot; ITS sequence analysis; EF sequence analysis; *Fusarium oxysporum*

Introduction

Panax notoginseng, one of the most distinguished traditional Chinese herbal medicines, is used for treatment of swelling and blood disorders, including local blood stasis and bleeding. It is known as "the king of ginseng" in the modern Chinese pharmacopeia, as it has a higher content of active ingredients than ginseng. In recent years, with the increase of *P. notoginseng* planting area and the reduction in crop rotation duration, the frequency and severity of *P. notoginseng* diseases have also increased significantly and caused more serious product losses, and they have become a serious obstacle to *P. notoginseng* production. Root rot is one of the most serious diseases of *P. notoginseng*, whose incidence each year can range from 5% to 70% or even higher. After infection by root rot, the leaves of *P. notoginseng* turn yellow, wilt to the ground and smell badly as they start to rot. Disease and pest control is an important part of *P. notoginseng* cultivation. Diseases mainly affect the crop's rhizomes, flowers and leaves, inhibiting plant growth, and lowering production. Root rot of *P. notoginseng* was first described in 2006, and reported to be caused by the fungus *Fusarium scirpi* (Miao *et al.,* 2006). A number of papers in the 1980s and 1990s on the *P. notoginseng* root rot pathogen reported mainly a fungus producing sickle-shaped spores. Since the cultivation history of most plant-based Chinese medicinal materials is short, the plants retain many wild characters. As a consequence, many of these Chinese medicinal plants inevitably suffer from serious harm from pests and pathogens when artificially cultivated in monocultures over a large area. Because many species of *Fusarium* produce spores with similar characteristics, it is difficult to accurately distinguish species using only the traditional morphological features. Combining morphological features and molecular methods together, on the other hand, has become important in identifying *Fusarium* species. Indeed, RFLP has successfully distinguished between *F. oxysporum* f. sp. *lycopersici* and f. sp. *radicis-lycopersici* (Attitalla *et al.,* 2004).

We focus here on *P. notoginseng* of Chinese herbal medicine, and the situation in *P. notoginseng* plantations in the city of Guiyang and some counties in the neighbouring southeast of Guizhou province from 2015 to 2016, where quite serious root rot was found. The disease progressed to its peak level from March to April and from June to August, with *P. notoginseng* plots exhibiting root rot incidence rates of more than 30%. As part of an effective strategy to manage *P. notoginseng* root rot in plantations, it was important to identify the pathogen unequivocally. We investigated the *P. notoginseng* root rot pathogen using morphological and molecular parameters, and investigated the factors affecting the frequency and severity of the disease, and the effect of natural and man-made environmental factors on disease severity, as the basis of recommendations for the prevention or control of *P. notoginseng* root rot.

Materials and Methods

Sample Collection and Pathogen Isolation

Diseased samples were collected from twenty-five examples of rhizomes rot on *P. notoginseng* from the city of Guiyang and some neighbouring counties of southeast Guizhou province between March and October 2015. Naturally-infected rhizomes were collected from the fields and the surrounding area. The rhizome samples were disinfected in 75% (v/v) ethanol for 1 min and then in 1% (v/v) sodium hypochlorite for 3 min followed by rinsing in sterilized water. After drying, tissue plugs (0.5 to1.0 cm in diameter) were taken from the juncture between diseased and healthy tissues and placed onto PDA in 90 mm diameter Petri dishes and incubated at 25°C and illuminated with photoperiod of 12 h dark/12 h light. Fifteen isolates were obtained from the mycelium which developed from the different diseased samples, and pure cultures were derived from each isolate and stored on PDA slopes at 4°C for consequent studies.

Investigation of Disease Occurrence and Frequency

Investigations into the occurrence and frequency of *P. notoginseng* root rot were carried out in the city of Guiyang and some counties of southeast Guizhou province from March 2015 to October 2016. Investigations involved mainly the time of disease occurrence, the symptoms of infected plants, the distribution of the disease, the frequency of incidence of the disease and the effects of infection on yield and quality of *P. notoginseng*. The sampling plot is 20, we collecting 100 samples every time and sampling every two weeks during the two years of investigation.

Pathogenicity Tests on Isolates

Koch's postulates: A pathogenicity test was conducted by inoculating the fungal isolate onto fresh rhizomes of *P. notoginseng* seedlings (2 years) *in vitro*, according to Koch's Postulates. Before inoculation, the rhizomes were disinfected by immersion in 75% (v/v) ethanol for 1 min, then rinsed in sterilized water, and then air-dried under sterile conditions in a laminar air-flow cabinet. The disinfected rhizomes were wounded with a sterilized scalpel and a conidium suspension (0.1 mL; 10^7 conidia per mL) was applied to the rhizomes of each seedling with a sterile syringe needle and the controls were inoculated with sterile water only. Inoculated seedlings were placed in 90 mm Petri dishes containing moistened paper towels and incubated at 25°C and illuminated with photoperiod of 12 h dark/12 h light for 7 days, and symptoms were noted. To fulfil Koch's postulates, diseased tissue from the surface-sterilized inoculated rhizome was plated onto PDA, and the fungus re-isolated for observation to confirm the characteristics of the voucher specimen. Experiments were repeated at least twice.

Single Spore Isolates

The Pathogenic fungal spores of root rot of *P. notoginseng* were scattered thinly over the water Joan, and then examination with the microscope for single spore on the surface of the plastic board in the water Joan, and transplanting it to cant PDA medium in sterile conditions, incubated at 25°C for 7 days. Then obtain a single cell strain colonies.

Morphological Characterization

Pathogen morphology was observed by microscopy. A 5 mm diameter plug from the colony was placed centrally onto a 90 mm PDA plate, followed by incubation at 25°C and a 12 h photoperiod, to observe the growth of hyphae. The isolates were identified initially by comparing morphological and cultural characteristics after 7 days. Spore size was determined by measuring the length and width of 30 to40 randomly selected conidia (Xiao *et al.*, 2011).

Molecular Identification

ITS sequence analysis: The fungal DNA extractions were conducted using the CTAB method (Zhang *et al.*, 2008). The PCR primers used for amplification of the rDNA ITS region were ITS1 (5′-TCCGTAGGTGAACCTGCGG-3) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3) (Wu *et al.*, 2009). PCR amplification was performed in a 25 μL reaction mixture containing 1 μL template DNA (30 ng), 0.5 μL each primer (10 μmol/L), 2.5 μL 10×PCR buffer, 2.5 μL $MgCl_2$ (25 mol/L), 0.5 μL dNTPs (10 mmol/L), 0.5 μL Taq polymerase (2.5 U/μL) and 17 μL ddH_2O. After a pre-denaturation step of 94°C for 5 min, 35 cycles were performed on a Bio-RAD PTC-200 thermocycler using the following conditions: denaturation at 94°C for 0.5 min was followed by annealing at 53°C for 0.5 min and extension at 72°C for 1.5 min, followed by a final extension of 72°C for 7 min. An aliquot (5 μL) of PCR product was loaded onto a 1.2% (w/v) agarose gel (containing ethidium bromide) for electrophoresis at 5V/cm and visualized under UV.A 100 bp DNA ladder was used as a size marker (TianGen, Beijing, China). Candidate fragments were visualized in agarose gel and purified by a DNA purification kit (TaKaRa, Shiga, Japan). Sequencing was carried out by Sinogenomax (Beijing, China). The rDNA ITS8S sequences were aligned and searched for from the GenBank database using the National Center for Biotechnology Information Blast Network Server (Nucleotide BLAST).

EF sequence analysis: The fungal DNA extractions were conducted using the CTAB method (Zhang *et al.*, 2008). The PCR primers for amplification of rDNA EF region were EF1-526F: (5′-GTCGTYGTYATYGGHCAYGT-3′) and EF1-1567R: (5-ACHGTRCCRATACCACCRATCTT-3′). PCR amplification was performed in a 25 μL reaction

mixture containing 1 μL template DNA (30 ng), 0.5 μL of each primer (10 μmol/L), 2.5 μL 10×PCR buffer, 2.5 μL MgCl$_2$ (25 mol/L), 0.5 μL dNTPs (10 mmol/L), 0.5 μL Taq polymerase (2.5 U/μL) and 17 μL ddH$_2$O. After pre-denaturation at 94°C for 5 min, denaturation at 94°Cfor 30 s was followed by annealing at 54-63°C for 50 s and extension at 72°C for 90 s, followed by a final extension at 72°C for 7 min, 46 cycles in total. Candidate fragments were visualized in agarose gel and purified using a DNA purification kit (TaKaRa).Sequencing was carried out by Sinogenomax. The rDNA EF sequences were aligned and similarity searches from the GenBank database were determined using the National Center for Biotechnology Information Blast Network Server (Nucleotide BLAST).

High throughput Sequencing

After DNA Extraction of DNA from the infected tissue of *P. notoginseng*, Building a library after the electrophoretic detection is qualified (Dai *et al*., 2010), the qualified samples was breaken into a 350 bp fragment random with Covaris, build the library with TruSeq DNA Sample Prep Kit shijihe, after repair of DNA fragments, plus PolyA tail and sequencing joint, then purification and PCR amplification and building library in the end (Wang *et al.*, 2010), then identify the species of fungi in the infected tissue of *P. notoginseng*.

Maximum Parsimony

Maximum parsimony (MP) analysis was performed using PAUP (Phylogenetic Analysis Using Parsimony) v.4.0b10 (Swofford, 2003). The trees were inferred by using the heuristic search option with tree bisection-reconnection (TBR) as the branch swapping algorithm and 1000 random sequence additions. Maxtrees were setup to 5000, branches of zero length were collapsed and all multiple parsimonious trees were saved. Descriptive tree statistics for parsimony tree length [TL], consistency index [CI], retention index [RI], rescaled consistency index [RC] and homoplasy index [HI] were calculated for the Maximum Parsimony Tree (MPT). The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications, each with ten replicates of random stepwise addition of taxa (Felsenstein, 1985).

Results

Disease Symptoms

Diseases of *P. notoginseng* that have been reported include root rot, black rot, round spot, epidemic disease and rust rot. The leaves on an infected *P. notoginseng* plant began to pale to yellow early in the infection process. When an infected plant was pulled out of the ground, the rhizome base appeared brown in colour, with small yellow rust-like spots

forming on the rhizome epidermis. The spots gradually expanded and converged, to form disease lesions with a near-circular, oval or irregular shape, slightly raised margin, and concave center. When serious, the disease lesion can spread to the whole rhizome, leading to dry rot internally, with the skin rotting from parts of the rhizome (Fig. 1). The root rot disease developed rapidly during June to August.

Pathogenicity Test

To confirm pathogenicity, conidial suspension was used as the inoculum. The incidence of rhizomes exhibiting typical root rot symptoms following inoculation with the re-isolated pathogen was 86.5%. Disease symptoms began to appear on the third day after inoculation. Leaf tissue showed wilting symptoms in the early stages, and the rhizomes died after 5 days. When the rhizome was pulled up, clear rust-like symptoms were visible on the rhizomes of *P. notoginseng*. The symptoms on the inoculated plants were very similar to those on naturally-infected plants; the control plants, inoculated with sterile water, did not exhibit any disease symptoms (Fig. 1). The fungus was re-isolated from the lesions of the diseased rhizomes and was identical to the original isolates. We obtain 10 tube single cell strain colonies by single spore isolates of dilution method, set aside in refrigerator of 4°C.

Morphological Characterization

In culture and in nature, the fungus produced three types of spores: small conidia, large conidia and chlamydospores. Characteristics of the three spore types were as follows: small conidium: colourless, single-celled, oval or kidney-shaped, 5-12 μm× 2-3.5 μm; large conidium: colourless, multi-celled, sickle-shaped, slightly curved, slightly pointed at both ends of the cell; most have three septa, 19.6-39.4 μm × 3.5-5.0 μm; chlamydospore: pale yellow, subsphaeroidal, with smooth thick spore walls (Fig. 2).

On PDA, aerial mycelium developed and formed velvet-like white colonies. Later, colonies became pink to purple, due to the presence of a large number of spores. Colonies are 3-5 mm high following incubation at 25°C. The isolated fungus was identified tentatively as *Fusarium oxysporum* based on morphological and cultural characteristics described in Booth's monograph (Booth, 1988) and in other descriptions previously reported.

Molecular Identification of the Fungal Isolates from Infected Notoginseng

Fungal rDNA ITS and EF sequences evolve at a faster pace than coding regions, so can be used to distinguish species in the same genus or even to identify intra-specific variants, such as formae speciales. rDNA ITS and EF sequences have been widely used in *Fusarium* identification, phylogeny and genetic relationship studies.

Fig. 1: The leaf (A) and rhizome (B) symptoms of root rot on naturally-infected *P. notoginseng*, *P. notoginseng* before inoculation (C), Leaf symptoms after pathogen inoculation (D). rhizome symptoms after pathogen inoculation (E,F)
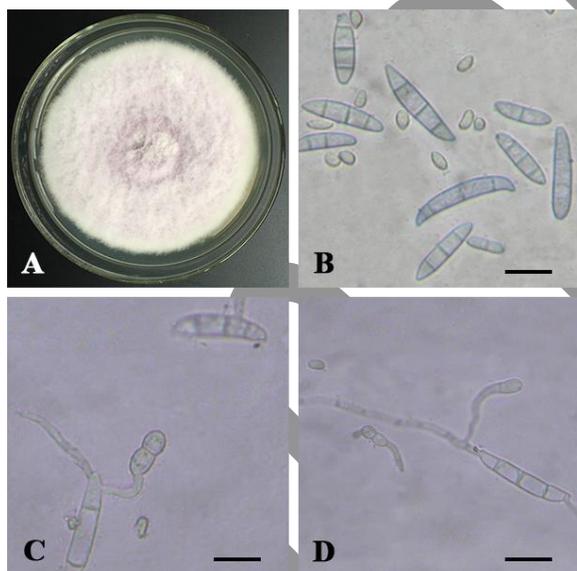

Fig. 2: Cultural characteristics: the growth of hyphae of *P. notoginseng* on PDA (A); the morphology of large conidia and small conidia of *F. oxysporum* (B); the spore production structures (C, D). Scale bar = 20 μm

ITS Sequence Analysis

DNA of fungi isolates from diseased *P. notoginseng* was amplified by PCR with primers for ITS1 and ITS4. The corresponding PCR-amplified region was the ITS rDNA sequence of regions 1 and 2, which also included the 5.8S

rDNA gene. The PCR product was approximately 490 bp for all isolates; high-quality sequences of approximately 490 bp from each isolate were used for analysis. The PCR product base sequence was compared by BLAST search in GenBank database. The ITS sequence of the fungi was most similar (100%) to *F. oxysporum* isolates (GenBank Accession No. KT896661).

EF Sequence Analysis

DNA of fungi isolates from diseased *P. notoginseng* was amplified by PCR with primers EF1-526F and EF1-1567R. The corresponding PCR- amplified region was the EF rDNA sequence of regions 1 and 2. The PCR product was approximately 970 bp for all isolates; high-quality sequences of approximately 970 bp for each isolate were used for analysis. The PCR product sequence was compared by BLAST search in GenBank database. The EF sequence of the fungi was most similar (99%) to *F. oxysporum* isolates (GenBank Accession No. JF740813).

High throughput Sequencing

After the sequencing data has been removed, data filtering in the first, obtain clean reads after removed the low quality data. Compare clean reads with the reference genome. The results indicate that, the quality of the samples was qualified, there are 10 species of fungi by High throughput sequencing, *Fusarium* is the majority, in which reads number of *Fusarium oxysporum* f. sp. lycopersici 4287 has 151, *Fusarium oxysporum* has 14, *Fusarium oxysporum* f. sp. cubense race 4 has 7, *Fusarium fujikuroi* has 46, *Fusarium verticillioides* has 8, *Rhizophagus intraradices* has 5, *Phialocephala scopiformis* has 4, uncultured fungus has 3, *Cladophialophora bantiana* has 3, *Metarhizium majus* ARSEF 297 has 2 (Fig. 3).

Maximum Parsimony

Phylogenetic analysis of ITS sequence data indicate that sanqi forms a separate branch is *F. oxysporum* (Fig. 4). In the ITS phylogenetic analyses, approval rating reached 79% values above the branches indicate maximum parsimony and maximum likelihood bootstrap≥79%. Thus, based on phylogenetic analysis, sanqi is considered to be *F. oxysporum.*

Investigation of Disease Occurrence and Frequency

P. notoginseng root rot forms under warm damp conditions. These conditions are conducive to many diseases of *P. notoginseng*, one of the most serious of which is *P. notoginseng* root rot. *P. notoginseng* root rot is a complex soil-borne disease. Infection involves a close relationship between the pathogen's virulence and environmental conditions such as temperature, humidity, light, cropping system and crop management method.
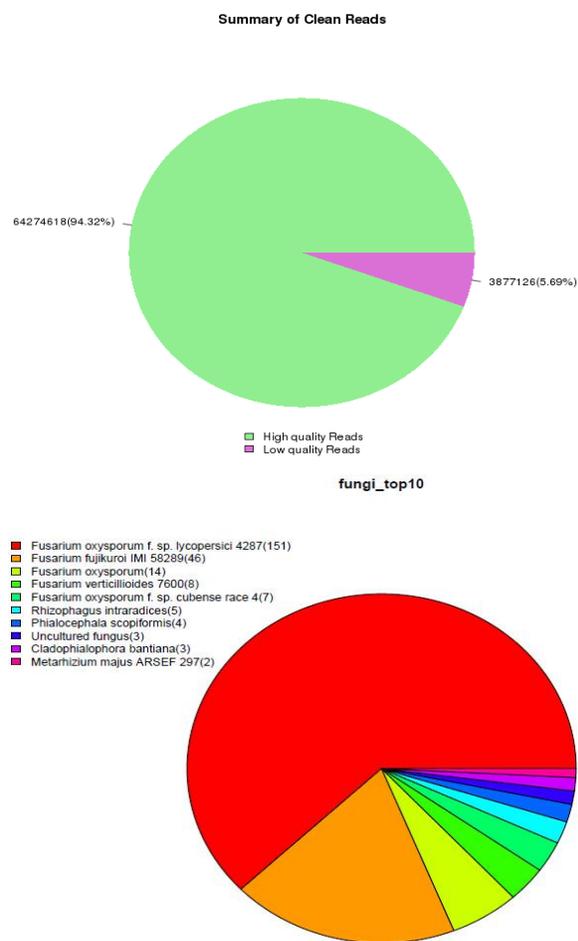
Summary of Clean Reads

64274618(94.32%)

3877126(5.69%)

☐ High quality Reads
☐ Low quality Reads

fungi_top10

■ Fusarium oxysporum f. sp. lycopersici 4287(151)
■ Fusarium fujikuroi IMI 58289(46)
■ Fusarium oxysporum(14)
■ Fusarium verticillioides 7600(8)
■ Fusarium oxysporum f. sp. cubense race 4(7)
■ Rhizophagus intraradices(5)
■ Phialocephala scopiformis(4)
■ Uncultured fungus(3)
■ Cladophialophora bantiana(3)
■ Metarhizium majus ARSEF 297(2)

Fig. 3: The quality inspection result of the naturally-infected *P. notoginseng* and the species of fungi in the sample
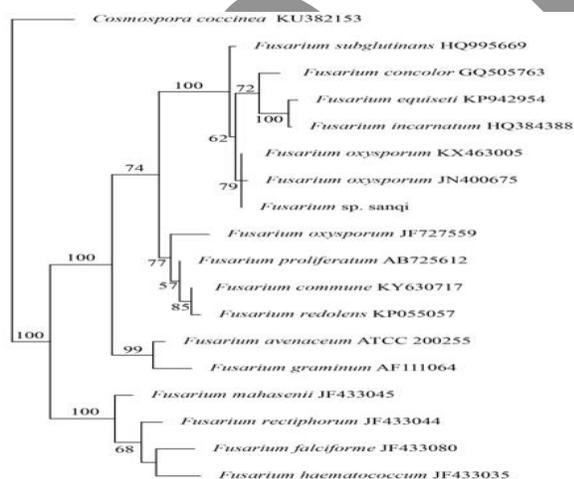

Fig. 4: Maximum likelihood (ML) majority rule consensus tree of combined ITS of sanqi sequence data based on MP, ML and Bayesi an analyses. Values above the branches indicate maximum parsimony and maximum likelihood bootstrap≥79%, (MPBS/MLBS)

Root rot incidence peaked twice in the *P. notoginseng* planting area of Guizhou province in 2015. The first peak appeared from March to April, with seedling root rot in this season requiring the relative humidity to be more than 80%. The second peak appeared from July to August; when relative humidity was more than 85% and the temperature above 20°C, root rot disease spread rapidly.

The results of the 2016 survey were basically identical to those of 2015. The first disease peak was also from March to April, while the second peak occurred 2-3 months after the start of the rainy season in July. Temperature was the main environmental factor influencing the disease, because July to August is the rainy season and the humidity increase associated with the rains was conducive to the development of the disease. *P. notoginseng* is a typical under-storey shade plant, and plantations should be set up under shady conditions, as plants stressed by high were particularly vulnerable to *P. notoginseng* root rot.

In the process of investigation, we found that *P. notoginseng* root rot was almost universal in *P. notoginseng* plantations, but that there were differences in the incidence of *P. notoginseng* root rot between different fields. The survey found that, when newly cultivated land was planted with *P. notoginseng*, the incidence of root rot was very low, but with the extension of *P. notoginseng* planting, the incidence and severity of root rot increased gradually; land with high incidence of the pathogen can result in yield losses of 20%. This is because the pathogen inoculum in the soil reproduced dramatically in the continuous presence of the host plant.

Discussion

The current study has isolated the causal pathogen from *P. notoginseng* root rot collected in Guizhou Province. *F. oxysporum* has been identified as the pathogen causing *P. notoginseng* root rot disease using traditional morphological methods combined with modern molecular biology techniques. Symptoms induced by *F. oxysporum* include wilt, root rot and decay. Hosts of *F. oxysporum* include melon, lettuce, strawberry and watermelons, and frequently different hosts are attacked by host-specific forma especiales of *F. oxysporum*.

Environmental conditions, such as humidity, temperature and light, are important factors determining how early in the season *P. notoginseng* root rot starts, the speed of disease development and the severity of the infection. The optimum temperature for root invasion by the pathogen lies between 20°C and 30°C. Temperature and relative humidity are the environmental factors key to disease development in China's *P. notoginseng* production areas; when the temperature is above 20°C, and the relative humidity is more than 95%, *P. notoginseng* root rot can be very serious. It has been reported that the occurrence and development of *P. notoginseng* root rot was closely related to the prevailing environmental conditions, with a

temperature of 20°C and a relative humidity greater than 95% being conducive to the occurrence and spread of the disease (Wang *et al.,* 1998). The conditions favouring *P. notoginseng* root rot are similar to the warm and humid conditions required for head blight floral infection on wheat caused by several *Fusarium* species (Parry *et al.,* 1995). Incident light levels also affects the occurrence and development of diseases.

Identification of the causal organism in *P. notoginseng* root rot is an important step in developing recommendations for the control of this potentially serious disease. As a result of our investigations, we recommend that it is necessary to plant *P. notoginseng* in restricted areas and to practice crop rotation. It has been reported that the occurrence of *P. notoginseng* root rot is linked to altitude, topography, soil type, rotation age and shade conditions. Reduction of light levels incident on *P. notoginseng* would have beneficial effects of both decreasing the occurrence and severity of root rot (and other diseases) and of increasing *P. notoginseng* production (Chen *et al.,* 2001). Shading could be achieved using netting or by intercropping *P. notoginseng* within a taller crop. Intercropping could have multiple benefits, shading *P. notoginseng* and reducing self-shading of the other crop, and providing greater soil microbial diversity, with the potential for greater biological control of the pathogen causing root rot.

A study showed that the occurrence of *P. notoginseng* root rot was associated with a particular ratio of different fertilizers, or with a single application of nitrogen fertilizer, which lead to the plant growing too tall and reducing the plant's ability to fight disease and inducing root rot; excessive fertilization will aggravate the occurrence of root rot (Wang *et al.,* 2007). The use of compound fertilizers is advisable with *P. notoginseng* cultivation as it can reduce root rot and increase production. As *P. notoginseng* root rot appears to be a typical soil-borne disease, soil disinfection maybe an appropriate management tool.

The relationship between the host and pathogen remains unclear, so it needs to be studied further. *Fusarium spp.* may be an important component of a damping-off/root rot disease complex under growing conditions (Punja, 1997). At present, there are no improved cultivars of *P. notoginseng* under cultivation, while selection within *P. notoginseng* germplasm resources and other breeding work is in its infancy, so it is difficult to control root rot by using the host disease resistance. Therefore, we need to strengthen research into the identification of disease resistance genes and resistance mechanisms in *P. notoginseng* as soon as possible.

In conclusion, we identified that *F. oxysporum* is the pathogen causing *P. notoginseng* root rot disease using traditional morphological methods combined with modern molecular biology techniques. In addition, we found that temperature, humidity, high light levels and lack of crop rotation were the main factors influencing the occurrence of *P. notoginseng* root rot.

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