

Research on the isolation and identification of black spot disease of *Rosa chinensis* in Kunming, China

Yanjie Li

Ministry of Education, Southwest Research Center for Engineering Technology of Landscape Architecture (State Forestry and Grassland Administration), Southwest Forestry University

Meiyang Pu

Ministry of Education, Southwest Research Center for Engineering Technology of Landscape Architecture (State Forestry and Grassland Administration), Southwest Forestry University

Yusi Cui

Ministry of Education, Southwest Research Center for Engineering Technology of Landscape Architecture (State Forestry and Grassland Administration), Southwest Forestry University

Gu Ju

Ministry of Education, Southwest Research Center for Engineering Technology of Landscape Architecture (State Forestry and Grassland Administration), Southwest Forestry University

Xi Chen

Ministry of Education, Southwest Research Center for Engineering Technology of Landscape Architecture (State Forestry and Grassland Administration), Southwest Forestry University

Louqin Wang

Ministry of Education, Southwest Research Center for Engineering Technology of Landscape Architecture (State Forestry and Grassland Administration), Southwest Forestry University

Hongzhi Wu

Yunnan Agricultural University

Chao Wang (✉ 47188127@qq.com)

Ministry of Education, Southwest Research Center for Engineering Technology of Landscape Architecture (State Forestry and Grassland Administration), Southwest Forestry University

Article

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Abstract

Through a survey of rose diseases in the South Tropical Garden in Kunming, China, it was found that black spot was the most common and serious disease of rose cultivated in the open air there, with an incidence of more than 90%. In this study, fungus isolation was performed on leaf samples of five black spot susceptible varieties of rose from the South Tropical Garden by tissue isolation. 18 strains of fungus were initially obtained, and 7 of them were finally identified to cause black spot symptoms on healthy leaves of rose after verification by Koch's rule. By observing the morphology of colonies and spores, and constructing a phylogenetic tree by combining molecular biology and multiple genes, 2 pathogenic fungus were identified, namely, *Alternaria alternata* and *Gnomoniopsis rosae*. *G. rosae* was the first pathogenic fungi of rose black spot isolated and identified in this study. The results of this study can provide a reference base for further research and control of the black spot disease of rose in Kunming.

Introduction

Rose is one of the most famous and popular of all flowers, China is the birthplace of the *Rosa chinensis*, which was well documented by Confucius when he traveled around the kingdoms at the end of the Spring and Autumn period in 413 B.C. The rose was spread from the Silk Road to Persia, Ceylon and other countries before the 12th century, and was highly praised and loved abroad as an endemic species for cultural exchange (Wu Lijuan, 2014). In China, rose is defined as the city flower by several cities and is widely used in urban landscaping, but in the process of planting and growing, it is vulnerable to diseases and insects, resulting in poor growth, leaf drop, wilting, and even death, which both affects the ornamental and reduces the economic value of the rose itself (Lou Xiyan et al, 2021). There are 10 reported species of rose diseases, of which seven are fungal: powdery mildew, black spot, downy mildew, gray mold, leaf mold, rust, and branch blight, respectively, among them, black spot is the most serious and has become a worldwide disease, which occurs commonly in most of the rose planting areas, especially in open field cultivation with a very high incidence (Feng Youren et al, 2015).

Rose black spot was first reported in Sweden in 1815, and in 1910 it was first reported in China. At present, black spot disease of rose occurs in all parts of China, and has become an important problem to be solved urgently in the production of rose. According to previous studies, there are 2 species of pathogens that cause black spot in rose: one is *Marssonina rosae* and the other is *Alternaria* spp. (Zhu JH et al, 2017). Abbas first reported in Pakistan that the pathogenic fungi causing black spot of rose are mainly the genus *Alternaria* (Abbas MF et al, 2017). In 2013, Xu et al. collected 15 leaf samples of rose with typical black spot symptoms in Xi'an, Xianyang, Baoji and Weinan, and identified the pathogenic fungus infesting black spot as *Marssonina rosae* after isolation and purification (Xu Lingling et al, 2013). Feng et al. collected diseased leaves of rose with black spot symptoms in the garden of Yuncheng College, after isolation and purification, morphological identification and molecular phylogenetic analysis, the pathogenic fungus causing black spot of rose was *Alternaria alternata* (Feng Baozhen et al, 2019).

The aim of this study was to accurately identify 2 pathogenic fungus isolated from the black spot diseased leaves of five rose species in the South Tropical Garden in Kunming, China, through morphological identification and molecular biology phylogenetic analysis combined with pathogenicity determination, which can provide a theoretical basis for subsequent research on the biological characteristics of rose black spot pathogens or for effective control of rose black spot disease in the future.

Materials And Methods

Experimental materials

In November 2021, a field survey of rose disease incidence was conducted in the South Tropical Garden in Kunming, China, and the leaf samples of five rose varieties ("Red Leonardo da Vinci", "Sweet Pretty", "Happy Carefree", "Benita" and "Home run") with obvious symptoms of black spot were randomly collected, and brought back to the laboratory for storage at 4 °C in the refrigerator.

Medium: potato dextrose agar medium (PDA): potato 200 g, glucose anhydrous 20 g, agar 17–20 g, distilled water volume to 1 L.

Reagents: Sangon Biotech Fungi DNA Rapid Extraction Kit, PCR primers (ITS1/ITS4, NS1/NS4, LSU1Fd/LR5, EF1-728F/EF1-986R, T1/T2), 2×Taq PCR Mix, nucleic acid dyes, DNA Marker DL2000, 5×TAE, etc.

Instruments: optical microscope, autoclave, constant temperature metal bath, low temperature centrifuge, PCR instrument, gel imaging electrophoresis instrument, constant temperature and humidity incubator, etc.

Experimental methods

Isolation and purification of pathogenic fungus

The tissue isolation (Zhang C. Y., 1992) was used to isolate the black spotted leaves of rose. After rinsing the diseased rose leaves, use a sterilized scalpel to cut 3×3mm pieces of tissue at the junction of diseased and healthy leaves in the ultra-clean bench, then put the tissue into 1% sodium

hypochlorite solution (5s), 75% alcohol (30s), finally rinse with sterile water 3 times, place on sterilized filter paper, dried and then inoculate the diseased tissue pieces onto the PDA medium with forceps. 3 tissue blocks were inoculated in each Petri dish, marked with number and date on the lid, sealed and incubated in a constant temperature and humidity incubator at 25°C. 3 groups of parallel controls were used for each experiment. After 2 days, the colonies were grown and immediately purified, after purification 2–3 times to obtain pure culture, inoculate it on PDA test tube slant, put it in 25°C constant temperature incubator to wait for colonies to grow full of medium, put it in the refrigerator at 4°C to be refrigerated.

Determination of pathogenicity of pathogenic fungus

ex vivo leaves inoculation: healthy leaves of five rose varieties("Red Leonardo da Vinci", "Sweet Pretty", "Happy Carefree", "Benita" and "Home run") were collected from the South Tropical Garden in Kunming,China in May 2022, and the variety numbers were recorded and brought back to the laboratory for processing in self-sealing bags. The healthy rose leaves were rinsed with tap water, then disinfected with 75% alcohol for 30s, then rinsed with sterile water 3 times and placed on sterile filter paper to dry. The fungus block were punched at the edge of the colonies with a 5mm diameter sterilized punch and set aside. Two layers of sterile filter paper were placed in a 9-cm diameter Petri dish and moistened with sterile water, the sterilized healthy rose leaves were placed on the filter paper, one compound leaf was placed in each Petri dish, 3 minimally invasive wounds were made on the small leaves with a sterilized scalpel, then a 5 mm mycelial block was inoculated on the wounds and the inoculation site was covered with a sterile wet cotton ball, 3 small leaves were treated with each compound leaf, and a control inoculation (inoculated with a 5 mm diameter PDA block) was set up, and each experimental group was repeated three times. After inoculation, the petri dishes were sealed with cling film and placed at room temperature, when symptoms appeared on the leaves, they were recorded and photographed immediately.

Inoculation in live leaves: the corresponding varieties of rose cuttings with relatively consistent leaf size were selected, and the surfaces of healthy rose leaves were first disinfected with skimmed cotton balls dipped in 75% alcohol, then the leaves were dipped in sterile water and wiped 3 times, and the parts to be inoculated were pricked with sterile inoculation needles, then a 5 mm mycelial block was inoculated on the wounds and the inoculation site was covered with a sterile wet cotton ball, 3 replicates were treated for each rose variety, 3 small leaves were treated for each compound leaf, and set up control inoculation (inoculated with a 5-mm diameter PDA block), and cover the inoculated whole compound leaves with plastic bags, observe and record daily and spray water to moisture the inoculated leaves. After the symptoms appear on the inoculated leaves, recorded and photographed immediately.

According to Koch's rule, the tissue of rose leaves at the junction of disease and health was taken for isolation and purification again, the re-isolated strain was compared with the original strain, and the same identification method as the original strain was used to test whether the isolated pathogenic fungus was consistent with the original pathogenic fungus.

Morphological identification of pathogenic fungus

The isolated and purified black spot disease pathogenic fungus of rose were inoculated onto PDA medium and incubated at 25°C for 7–10 d. The colony morphology, color and growth rate were observed and recorded. Slides were prepared by picking mycelium with an inoculating needle, and morphological characteristics such as mycelium, color, spore-producing structure, conidia and conidia were observed under the optical microscope and photographed and recorded.

Molecular biology and phylogenetic identification of pathogenic fungus

The pathogenic fungus were inoculated on PDA medium and incubated for 7 d, after the mycelium had grown all over the dishes, the total genomic DNA of the test strains was extracted and PCR amplification was performed using the fungal universal primers ITS(ITS1/ITS4),SSU(NS1/NS4),LSU(LSU1Fd/LR5),TEF(EF1-728F/EF1-

986R) and TUB(T1/T2) (Table <link rid="tb3">1</link>–1), and the reaction system is shown in Table 1–2; the PCR amplification reaction procedure is shown in Table 1–3. The amplified PCR products were detected by electrophoresis on a 1% agarose gel, and if there were single and clear bands, sent it to Biotech Bioengineering Ltd. for sequencing. The sequencing results were analyzed by sequence comparison on the NCBI website using the BLAST tool, and reference to Muhammad Farhan(Muhammad Farhan,2021) and Ning Jiang(Jiang N et al,2021), the known sequences with high homology in GenBank were searched online as model strains. Based on the sequences of multiple gene combinations of rDNAITS, SSU, LSU, TEF1, and LSU, a phylogenetic tree was constructed using the Maximum Likelihood Method using MEGA11.0 software to analyze the kinship of the black spot pathogenic strains of rose with the model strains and finally determine their taxonomic status(Xu CN et al,2015; Zhou Y et al,2015).

Results

Symptoms of black spot disease of rose

Through the survey on rose disease in the South Tropical Garden in Kunming, China, it was found that black spot was the most widespread and serious, with an incidence of over 90%. Black spot disease mainly infects the leaves of rose (Fig. 1). At the beginning of the disease, brown spots appear on the front side of the leaves, with the aggravation of the disease, the spots gradually expand into round or irregular spots, the spots are

purple-brown to dark brown; late in the disease, the tissue around the spots turns yellow, the lower and middle leaves of the plant all fall off, leaving only the top few leaves.

Isolation and determination of the pathogenicity of the black spot pathogen fungus of rose

18 fungal strains were isolated and purified from samples of diseased leaves of five different rose species ("Red Leonardo da Vinci", "Sweet Pretty", "Happy Carefree", "Benita" and "Home run"), these fungus mainly belonged to *Gnomoniopsis*, *Alternaria* and *Nigrospora* after DNA extraction, ITS amplification and sequencing, and preliminary comparison at NCBI. All fungus were inoculated separately on the ex vivo healthy rose leaves in the laboratory, and some of leaves started to show symptoms around 5 d, among which the healthy rose leaves inoculated with the strains of *Alternaria* and *Gnomoniopsis* spp. showed the most obvious symptoms (Table 2 – 1), while the other strains showed almost no symptoms in the ex vivo leaves after 7 d of inoculation. TMR3-3 of the genus *Gnomoniopsis* and KLWY1-6-2 of the genus *Alternaria*, which caused disease in ex vivo healthy rose leaves, were selected as representative strains for inoculation in live leaves, and black spot disease symptoms appeared on the leaves around 7 d (Table <link rid="tb5">2</link>– 2). The pathogenic parts of inoculated rose leaves were isolated again, and the same identification method as the original strains was used. The final strains obtained could all correspond to the original strains, so TMR1-1-2, HSDFQ3-7-1, HSDFQ2-4, DDBX2-13, KLWY1-6-2; XP1-6, TMR3-3 are all pathogenic fungus that infest black spot of rose.

Morphological identification of the pathogenic fungus of black spot disease of rose

The five strains of TMR1-1-2, HSDFQ3-7-1, HSDFQ2-4, DDBX2-13, KLWY1-6-2 of *Alternaria* spp. were incubated on PDA medium at 25°C for 7–10 d. The morphology and color were consistent, with the initial colonies being grayish white on the front, then gradually turning grayish brown and blackish brown on the back, and the colonies growing over the entire petri dish for about 10 d (Fig. 2-A, B). DDBX2-13 was selected as a representative strain and observed under the microscope, the conidia were chained, ovate, inverted pear-shaped or inverted clavate, brownish, with 0–3 longitudinal septa, 2–5 transverse septa, slightly narrowed at the separation, individual with columnar short beak, spore size was 12.1–41.0 $\mu\text{m} \times 7.0\text{--}19.0 \mu\text{m}$; conidial peduncle was erect or curved, cespitose, with branches; mycelium was vigorous and velutinous, colorless, non-septate, and about 2.1–3.0 μm in diameter (Fig. 2-C, D). Referring to the *Handbook of Fungal Identification*(Wei Jingchao, 1979), the pathogenic fungus was initially identified as the genus *Alternaria* based on morphological characteristics. 2 fungal strains of the genus *Gnomoniopsis* (XP-6, TMR3-3) were cultured at a constant temperature of 25°C in PDA medium, and the morphological characteristics were also consistent. The fungus grows in a ring, the initial mycelium is pure white, then the inner ring of mycelium gradually becomes gray-green, the middle ring is light green, the outermost ring is gray-white; later the colony as a whole is gray-green, the aerial mycelium is sparse, and finally the whole colony is ring-shaped and produces a large number of conidiophores with gray-green to black bases, the conidium is yellowish when it overflows; the conidium is fusiform, transparent without septum, the surface is smooth, with droplets, the tip is thinner than the bottom; the conidium is bottle-stemmed or clavate, transparent, smooth, the tip is thinner than the bottom (Fig. 3-A, B); with droplets, spores 7.5–10 $\times$ 3.5–4 μm ; spore-producing cells are bottle-stemmed or clavate, degenerated from conidial peduncle, transparent, smooth, tip finer than the bottom.(Fig. 3-C, D)

Molecular identification and phylogenetic analysis of pathogenic fungus of black spot disease of rose

Genomic DNA of five strains of the genus *Alternaria* (TMR1-1-2, HSDFQ3-7-1, HSDFQ2-4, DDBX2-13, KLWY1-6-2) was amplified by PCR using the fungal universal primers NS1/NS4, LSU1Fd/LR5, while two strains of the genus *Gnomoniopsis* (XP1-6, TMR3-3) were amplified by ITS (ITS1/ITS4), TEF (EF1-728F/EF1-986R) and TUB (T1/T2), and single and clear bands were obtained after electrophoretic detection on 1% agarose gels. The above PCR amplification products were sent to the Kunming Branch of Beijing DynaScience Biotechnology Co., Ltd. for sequencing, and the sequencing results were uploaded to the NCBI website to obtain the registration numbers. The phylogenetic tree was constructed using the neighbor-joining method with MEGA11.0 software, and the results showed that the strains DDBX2-13, KLWY1-6-2, HSDFQ3-7-1, TMR1-1-2, HSDFQ2-4 clustered in one branch with *Alternaria alternata* (Fig. 4); XP1-6 TMR3-3 clustered in one branch with *Gnomoniopsis rosae* (Fig. 5). Finally, combined with the morphological characteristics identification results, *Alternaria alternata* and *Gnomoniopsis rosae* were identified as the pathogenic fungus of black spot of rose.

Discussion And Conclusion

In this study, 18 morphologically distinct fungal strains were isolated from the black spot disease-infested leaf samples of five rose varieties("Red Leonardo da Vinci", "Sweet Pretty", "Happy Carefree", "Benita" and "Home run") through morphological identification, molecular biology identification combined with pathogenicity determination, and a total of seven black spot pathogenic strains were obtained after verification by Koch's rule. The mycelium, spore-producing structure, conidia and other structures of these seven pathogenic strains were observed morphologically under a 40x microscope, and finally the pathogenic DNA was extracted and sequenced by PCR amplification using the fungal universal primers ITS, SSU, LSU, TEF1 and TUB, and the phylogenetic tree was constructed by NCBI sequence comparison and downloading the

pattern strain multi-gene association in GenBank, and finally TMR1-1-2, HSDFQ3-7-1, HSDFQ2-4, DDBX2-13 and KLWY1-6-2 were identified as *Alternaria alternata*; XP1-6 and TMR3-3 were identified as *Gnomoniopsis rosae*.

The results of most of the existing studies on black spot of rose show that the pathogenic fungus is mostly *Marssinia rosae*, which is different from the results of the our study. Abbas, Peihong Fang et al. found the pathogenic fungus of black spot of rose as *Alternaria*, on PDA plates, the colonies started as grayish white and gradually became black or brown, and the conidia were septate, ovate, pear-shaped or rod-shaped (Peihong Fang et al, 2020), which is consistent with the morphology of *A. alternata* in this study. The other pathogenic fungus, *Gnomoniopsis rosae*, isolated and identified in this study, has not been reported in the field of research on black spot of *Rosa chinensis*. The reason for this may be due to the different geographical locations and climatic conditions of different rose varieties cultivated in different regions, so there are also differences in the species of black spot pathogens.

Gnomoniopsis rosae was first reported as a new record species in China in 2022 by Jiang, N. et al (Jiang N et al, 2022), who also reported that rose was a new host record for *G. rosae*. However, in contrast to the direction of our experimental study, *G. rosae* was isolated from healthy branches of the rose by Ning Jiang et al. and used as endophytic fungus for subsequent studies on the identification and characterization of species of the genus *Gnomoniopsis*. In this study, *G. rosae* was isolated from the leaf samples with black spot of two rose varieties ("Sweet Pretty" and "Home run"), which were verified by Koch's law and identified morphologically and molecularly phylogenetically, and finally identified as the pathogenic fungus of rose black spot disease. The hosts of the fungus of the genus *Gnomoniopsis* are mainly plants of the families Crustaceae and Rosaceae, and 25 species of *Gnomoniopsis* have been reported, of which 10 species are distributed in China, 9 species have been reported on plants of the family Crustaceae, and one has been reported on *G. rosae* of the family Rosaceae (Jiang N et al, 2021). Several species within the genus *Gnomoniopsis* have been reported as important plant pathogens (Stevanović M et al, 2019; Visentin I et al, 2012), e.g. *G. daii* has been reported as the causal agent of leaf spot and solid rot of Chinese chestnut (Jiang N et al, 2021; Jiang N et al, 2019); *G. fragariae* caused leaf spot disease of strawberry (Udayanga D et al, 2021); *G. chinensis* caused severe ulcer disease of chestnut in Hebei, causing serious economic losses locally (Jiang N et al, 2020); *G. clavulata* caused leaf spot of white oak and red mistletoe in North America (Sogonov M V et al, 2007); *G. smithogilvyi* caused severe solid rot of European chestnut in Europe, Australia and India (Lione et al, 2019; G Shuttleworth L A et al, 2016; Shuttleworth L A et al, 2017).

In this study, a new pathogenic fungi, *G. rosae*, belonging to the phylum Ascomycota, order Diaporthales, family Gnomoniaceae, and genus *Gnomoniopsis*, was identified for the black spot of rose. This is the first report of *G. rosae* causing black spot of *G. chinensis*. The identification results can provide a reference basis for further research and control of black spot of rose.

Declarations

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IUCN Policy Statement

Experimental studies and field research on plants (cultivated or wild), including the collection of plant material, must comply with relevant institutional, national and international guidelines and legislation. We will strictly adhere to the IUCN Policy Statement on Research on Endangered Species and the Convention on Trade in Endangered Species of Wild Fauna and Flora. All specimens were collected with permission from South Tropical Garden, Kunming. We confirm compliance with the IUCN policy for plant.

Conflict of interest

The authors declare that they have no conflict of interest.

Data Availability Statement

The data that support the findings of this study are openly available in the supplementary material of this article.

References

1. Abbas MF, Aziz-ud-Din, Rafique K, Qadir A, Rashid A, Qamar MI, Rafique M, Gleason ML. 2017. First report of *Alternaria* black spot of rose caused by *Alternaria alternata* in Pakistan. *Plant Disease*, 101(9): 1676

2. Carbone I, Kohn L M. A method for designing primer sets for speciation studies in filamentous ascomycetes *Cochliobolus*[J]. *Mycologia*, 1999, 91(3): 553-556.
3. Crous P W, Braun U, Wingfield M J, Wood A R, Shin H D, Summerell B A, Alfenas A C, Cumagun C J R, Groenewald J Z. Phylogeny and taxonomy of obscure genera of microfungi[J]. *Persoonia Mol Phylogeny Evol Fungi*, 2009, 22(1): 139-161.
4. Feng Youren, Liu Baosheng, Bai Penghua. Identification and biological characterization of a novel leaf blight pathogen of rose in Tianjin [J]. *Northern Horticulture*, 2015(14): 125-129.
5. Feng Baozhen, Li Peiqian. Identification of pathogenic bacteria and preliminary screening of indoor agents for black spot disease of rose [J]. *Journal of Plant Protection*, 2019.46(05): 1147-1154. doi:10.13802/j.cnki.zwbhxb.2019.2018149.
6. Jiang N, Xue H, Park CHG, Li Y. Identification and characterization of species of the genus *Hibiscus*[J]. *Journal of Terrestrial Ecosystems and Conservation*. doi: 10.12356/j.2096-8884.
7. Jiang N, 2021. Taxonomic study of Chestnut cysticercus in China [D]. Beijing: Beijing Forestry University.
8. Jiang N, Fan X L, Tian C M, 2021a. Identification and characterization of leaf-inhabiting fungi from *Castanea* plantations in China[J]. *Journal of Fungi*, 7(1):64.
9. Jiang N, Tian C M, 2019. An emerging pathogen from rotted chestnut in China: *Gnomoniopsis daii* sp. nov.[J]. *Forests*, 10(11): 1016.
10. Jiang N, Liang L Y, Tian C M, 2020. *Gnomoniopsis chinensis* (Gnomoniaceae, Diaporthales) A new fungus causing canker of Chinese chestnut in Hebei Province, China[J]. *MycologyKeys*, 67: 19-32.
11. Jiang N, Voglmayr H, Bian D R, et al. Morphology and phylogeny of *Gnomoniopsis* (Gnomoniaceae, Diaporthales) from fagaceae leaves in China[J]. *Journal of Fungi*, 2021, 7(10): 792.
12. Lione G, Danti R, Fernandez-Conradi P, et al, 2019. The emerging pathogen of chestnut *Gnomoniopsis castaneae*: the challenge posed by a versatile fungus[J]. *European Journal of Plant Pathology*, 153(3): 671-685.
13. Lou Xiyang, Wang Xinyang, Pei Dongli. Identification and biological characteristics of pathogenic bacteria of anthracnose of rose in Shangqiu, Henan [J]. *Jiangsu Agricultural Science*, 2021, 49(22): 116-121. doi:10.15889/j.issn.1002-1302.2021.22.020.
14. Muhammad Farhan. Phylogenetic relationships of *Streptomyces* spp. fungi on medicinal plants in Southwest China[D]. Guizhou University, 2021. DOI:10.27047/d.cnki.ggudu.2021.000611.
15. O'Donnell K, Nirenberg HI, Aoki T, et al. A Multigene phylogeny of the *Gibberellafujikuroi* species complex: Detection of additional phylogenetically distinct species [J]. *Mycoscience*, 2000, 41:61–78.
16. Peihong Fang, Shaochuan Shi, Xintong Liu, Zhao Zhang. First report of *Alternaria* black spot of rose caused by *Alternaria alternata* in China[J]. *Journal of Plant Pathology*, 2020, 102(1).
17. Stevanović M, Ristić D, Živković S, et al, 2019. Characterization of *Gnomoniopsis idaeicola*, the causal agent of canker and wilting of Blackberry in Serbia[J]. *Plant Disease*, 103(2): 249-258.
18. Sogonov M V, Castlebury L A, Rossman A Y, White J F, 2007. The type species of *Apiognomonina*, *A. veneta*, with its *Discula* anamorph is distinct from *A. errabunda*. *Mycological Research*, 111(6): 693-709.
19. Shuttleworth L A, Walker D M, Guest D I, 2016. The chestnut pathogen *Gnomoniopsis smithogilvyi* (Gnomoniaceae, Diaporthales) and its synonyms[J]. *Mycotaxon*, 130(4): 929-940.
20. Shuttleworth L A, Guest D I, 2017. The infection process of chestnut rot, an important disease caused by *Gnomoniopsis smithogilvyi* (Gnomoniaceae, Diaporthales) in Oceania and Europe[J]. *Australasian Plant Pathology*, 46(5): 397-405.
21. Udayanga D, Miriyagalla S D, Manamgoda D S, et al, 2021. Molecular reassessment of diaporthalean fungi associated with strawberry, including the leaf blight fungus, *Paraphomopsis obscurans* gen. et comb. nov. (Melanconiellaceae)[J]. *IMA Fungus*, 12(1): 1-21.
22. Visentin I, Gentile S, Valentino D, et al, 2012. *Gnomoniopsis castanea* sp. nov. (Gnomoniaceae, Diaporthales) as the causal agent of nut rot in sweet chestnut[J]. *Journal of Plant Pathology*, 94(2): 411-419.
23. Vilgalys R, Hester M. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species[J]. *J Bacteriol*, 1990, 172(8).
24. Wu Lijuan. Research on the culture of rose [D]. China Academy of Forestry Science, 2014.
25. White T J, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols*[J]. Elsevier, 2(3): 315-322.
26. Wei Jingchao. Handbook of fungal identification [M]. Shanghai: Shanghai Science and Technology Press, 1979: 424-425.
27. Xu CN, Zhang HJ, Zhou ZS, Hu TL, Wang ST, Wang YN, Cao KQ. 2015. Identification and distribution of Botryosphaeriaceae species associated with blueberry stem blight in China. *European Journal of Plant Pathology*, 143(4): 737-752.
28. Xu Lingling, Tao Guirong, Yao Wuwei, Li Ying, Wang Xiong, Guo Bin. Isolation of endophytic fungi and screening of active strains against black spot disease in rose[J]. *Anhui Agricultural Science*, 2013, 41(15):6699-6701. doi:10.13989/j.cnki.0517-6611.2013.15.104.

29. Zhu JH, Zhang HZ, Chen QIR, Xiong XY, Zhong XH, Li YL. Research progress on the occurrence and damage of black spot disease and resistance breeding of rose [J]. Journal of Hunan Agricultural University (Natural Science Edition), 2017, 43(01):47-51.DOI:10.13331/j.cnki.jhau.2017.01.009.
30. Zhang C. Y. Fungal diseases of ornamental plants [M]. Chengdu: Sichuan Science and Technology Press, 1992.
31. Zhou Y, Gong GS, Cui YL, Zhang DX, Chang XL, Hu RP, Liu N, Sun XF. 2015. Identification of Botryosphaeriaceae species causing kiwifruit rot in Sichuan Province, China. Plant Disease, 99(5):699-708.

Tables

Table 1-1 Primers used for PCR amplification of black spot pathogens in rose

Gene	Primer	Primer sequence	Reference
ITS	ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	White et al.(1990)
	ITS4	5'-TCCTCCGCTTATTGATATGC-3'	
SSU	NS1	5'-GTAGTCATATGCTTGTCTC-3'	White et al.
	NS4	5'-CTTCCGTCAATTCCTTTAAG-3'	
LSU	LSU1Fd	5'-GRATCAGGTAGGRATACCCG-3'	Crous et al.(2009)
	LR5	5'-TCCTGAGGGAACTTCG-3'	Vilgalys and Hester(1990)
TEF1	EF1-728F	5'-CATCGAGAAGTTCGAGAAGG-3'	Carbone and Kohn.(1999)
	EF1-986R	5'-TACTTGAAGGAACCCTTACC-3'	
TUB	T1	5'-AACATGCGTGAGATTGTAAGT-3'	O'Donnell et al.(2000)
	T2	5'-TAGTGACCCTTGGCCCAGTTG-3'	

Table1-2 PCR antithesis system

ddH ₂ O	9.5μl
2×Taq PCR Mastermix	12.5ul
Upstream primers(10μmol/L)	1ul
Downstream primers(10μmol/L)	1μl
DNA Template(100 ng/ul)	1μl
Total volume	25μl

Table1-3 PCR protocol for primer pairs

primers	Pre-mutability temperature time (°C) (min)		mutability		annealing		extension		extension temperature		number of cycles
			temperature (°C)	time (s)	temperature (°C)	time (s)	temperature (°C)	time (s)	(°C)	time (min)	
ITS1/ITS4	94	4	94	30	61	30	72	90	72	10	35
NS1/NS4	94	5	94	30	48	30	72	90	72	7	35
LSU1Fd/ LR5	94	5	94	30	48	30	72	90	72	7	35
EF1-728F/ EF1-986R	94	5	94	30	52	30	72	45	72	7	40
T1/T2	95	4	95	30	61	30	72	60	72	10	35

Table 2-1 Symptoms of 7-d incidence of pathogenic fungus on healthy moonflower leaves inoculated in vitro

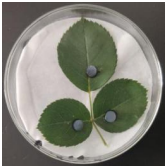
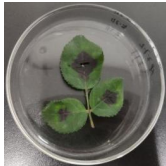
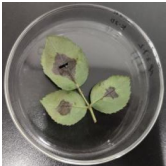
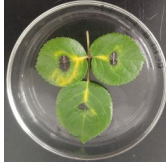
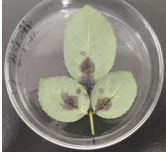
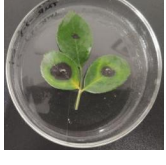
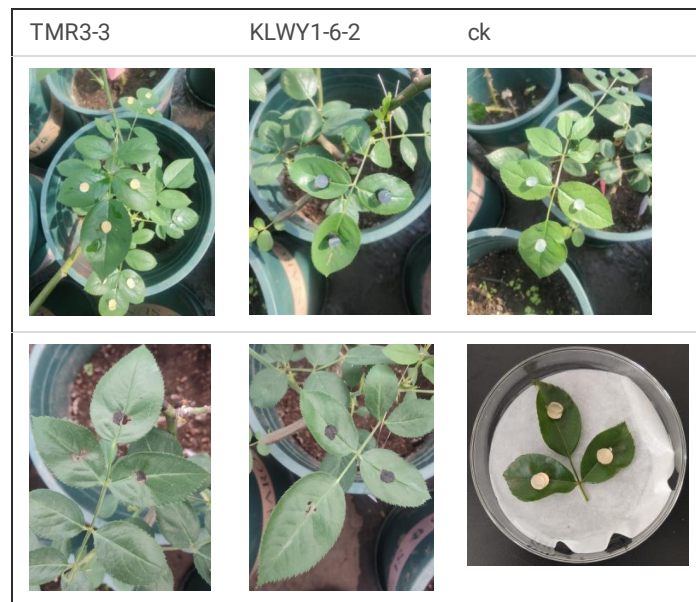
strain number	host	genus	Inoculation of pathogenic fungus	Front	Back
TMR1-1-2	"Sweet Pretty"	<i>Alternaria</i>			
HSDFQ3-7-1	"Red Leonardo da Vinci"	<i>Alternaria</i>			
HSDFQ2-4	"Red Leonardo da Vinci"	<i>Alternaria</i>			
KLWY1-6-2	"Happy Carefree"	<i>Alternaria</i>			
DDBX2-13	"Benita"	<i>Alternaria</i>			
XP1-6	"Home run"	<i>Gnomoniopsis</i>			
TMR3-3	"Sweet Pretty"	<i>Gnomoniopsis</i>			
ck	—	PDA			

table2-2 Symptoms of 7d inoculation of healthy leaves with pathogenic fungus in vivo



Figures



Figure 1

Field symptoms and diseased leaves of black spot of rose

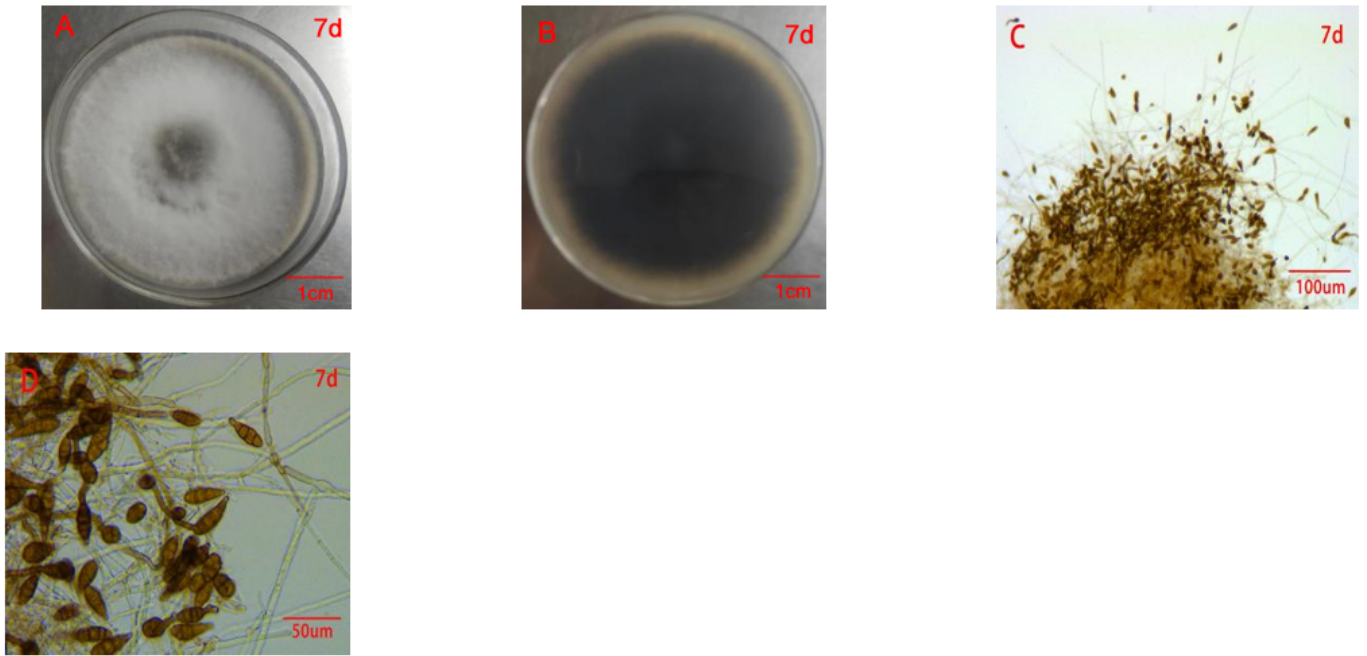


Figure 2

Colony and spore morphology of *Alternaria alternata*.

A. alternata grow on PDA medium for 7 days. A.front, B.back

Ascocarp under anatomical microscope was cultured for 7 days C,D

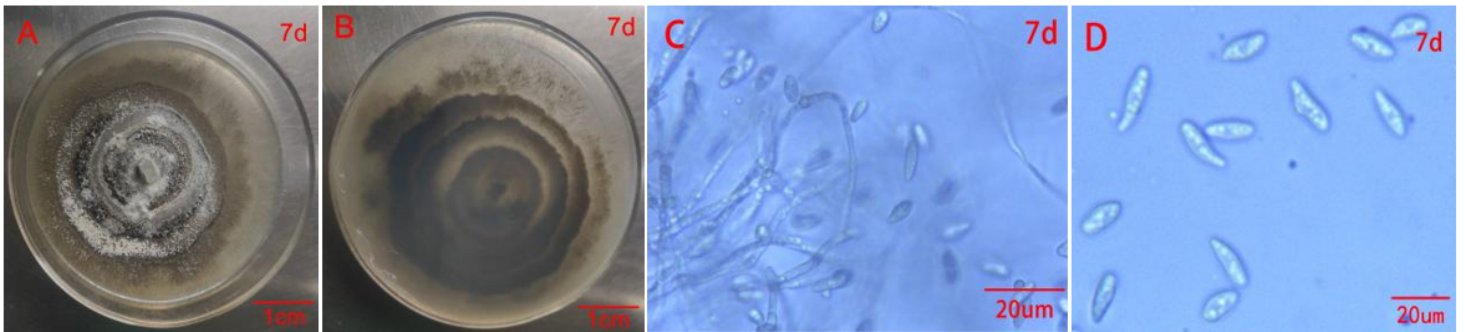


Figure 3

Colony and spore morphology of *Gnomoniopsis rosae*

G. rosae grow on PDA medium for 7 days. A.front, B.back

Ascocarp under anatomical microscope was cultured for 7 days C,D

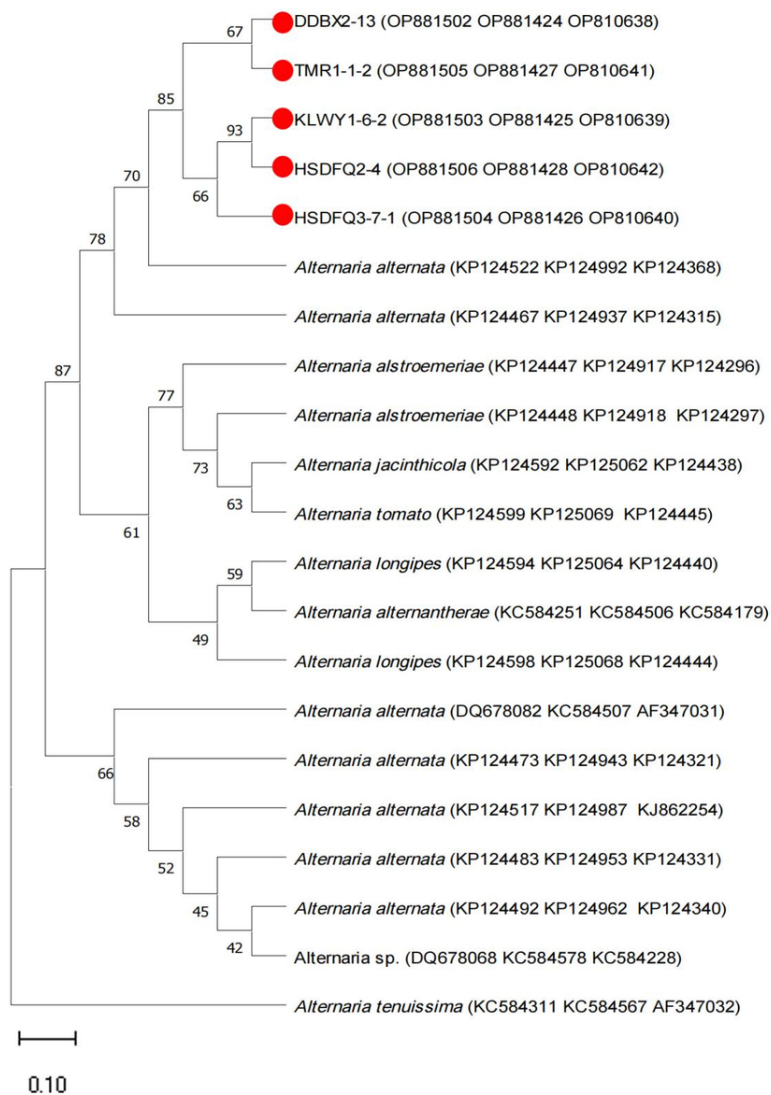


Figure 4

The maximum likelihood phylogenetic tree from combined LSU, SSU and ITS dataset of *Alternaria alternata* and its closed related species. (The accession numbers of LSU, SSU and ITS genes are shown in parentheses in that order)

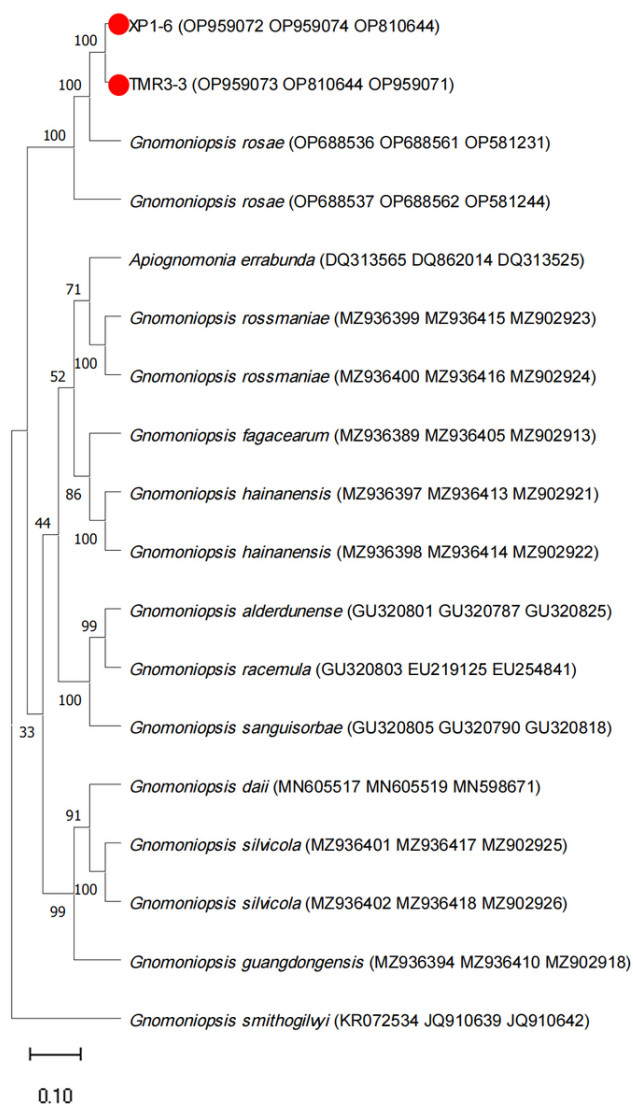


Figure 5

The maximum likelihood phylogenetic tree from combined TEF1, TUB and ITS dataset of *Gnomoniopsis rosae* and its closed related species.(The accession numbers of TEF1, TUB and ITS genes are shown in parentheses in that order)