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Morpho-molecular Identification and Chemical control of *Alternaria alternata*, causing leaf blight of *Gardenia jasminoides* Eills in China

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Abstract *Gardenia jasminoides* Eills is a traditional aromatic and medicinal plant that is widely cultivated in China. In 2020, a severe leaf disease on *Gardenia* was observed in a growing herbal medicine area in Jian County, of Jiangxi Province, China. The causal agent was identified as *Alternaria alternata* (Fr.) Keissler. by amplification and sequencing the internal transcribed spacer (ITS) region, followed by phylogenetic analysis. Koch's postulates were confirmed by a pathogenicity test conducted with healthy gardenia, including reisolation and identification. To our knowledge, this is the first report of leaf blight caused by *Alternaria alternata* on *Gardenia jasminoides* Eills in JiangXi, China. PDA plate bacteriostatic experiment results showed that: Hexaconazole had the best inhibiting effect, which the EC₅₀ was 14.45 ppm. Therefore, the

results are preliminary but promising for future field applications.

Keywords leaf blight • *Alternaria alternata* • chemical control

Introduction

Gardenia jasminoides Eills, belonging to the family Rubiaceae, is a famous ornamental plant widely distributed worldwide. The central officinal part of *G. jasminoides* is its dried ripe fruit. Chemical composition analysis finds multiple secondary metabolites in the fruit of *G. jasminoides*, like iridoid glycosides, crocin and crocetin, which have broad-spectrum anti-inflammatory properties, anti-cancer effects even can treat cerebral diseases (Zhang et al. 2021). The crocus sativus glycoside in gardenia fruit can still be used to produce gardenia red, gardenia yellow, gardenia blue, three kinds of pigment, as a natural colourant widely used in the food industry (Wu et al. 2021). There is a great demand for gardenia in China due to its wide application in the medicine and food industry. As wild gardenia was not enough to meet the demand of many people, people began to cultivate gardenia on a large scale to produce more gardenia for people to use. However, the large-scale planting of gardenia resulted in the decline of disease resistance and the frequent occurrence of disease, which seriously affected the yield and quality of gardenia.

Leaf blight is a common disease in rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), apple (*Malus pumila* Mill.), maize (*Zea mays* L.) (Mehl et al.

2021), garlic (*Allium sativum* L.) (Gálvez et al. 2016) and other plants. Once it happens, it is easy to break out and become a disaster, which seriously affects the growth and yield of plants and causes great economic losses. This is the first time that the leaf blight was observed in the gardenia planting base in Jiangxi province. The disease begins to infect the leaf margin, with irregular spots and later a large area of dry up, leading to plant death in severe cases. At present, the task is to identify the pathogen causing leaf blight and search for drugs to control the disease.

Materials and methods

Isolates Used and Morphological Observation

Survey of herbal medicine growing areas in Jian country (114°09'E 27°07'N) was carried out to assess the prevalence of the disease. The fungi from the infected samples were isolated using routine tissue isolation method. Leaves were disinfected with 0.5% NaClO for 5 min and washed twice with sterile distilled water. Small pieces from the edges of diseased tissue were placed on potato dextrose agar (PDA) medium. The isolated pathogens were purified using hyphal tip technique. After culturing for two days at 30 °C, transferred to a new PDA plate followed by incubation at 30 °C for five days. Emerging colonies were transferred several times by the hyphal tip method until and pure cultures were obtained. After five days, the morphological features of the culture of each isolate were

studied on PDA at 30 °C . The sterile cultures were stored at 4 °C in a refrigerator.

Pathogenicity test

The purified fungi were inoculated on healthy leaves and cultured in darkness in a constant temperature incubator at 30 °C , with control leaves instead of being inoculated using fungi-free PDA. After 48 hours, the incidence was observed, recorded the symptoms and characteristics of the injury were and took photos were for preservation. To complete Koch's postulates, fungi were isolated from diseased parts of inoculated Leaves, using the same method as when pathogenic bacteria were isolated. The morphological characteristics of the isolated microorganisms were observed and recorded.

Molecular identification

Mycelium was used to extract the genomic DNA by following the CTAB method described by Möller et al. primers ITS1 and ITS4 were used to amplify the ITS region of DNA. The polymerase chain reaction (PCR) was performed in an iCycler Thermal Cycler in a final volume of 50 µL containing 25 µL of 2 × PCR mix, 2 µL of each primer (10 µM), 2 µL of DNA (10 ng), and 19 µL of double-distilled H₂O. The PCR conditions include the 4 min duration for the initial denaturation at 95 °C ; followed by 35 cycles of 60 s at 94 °C , 60 s at 54 °C , and 60 s at 72 °C ; and a final extension of 10 min at 72 °C . The resulting PCR products were analyzed

by gel electrophoresis in a 1% agarose gel in 1 × Tris-borate-EDTA buffer and sequenced by Sangon Biotech Co., Ltd. (Shanghai, China). The amplified sequence was compared with the other sequences found in the National Center for Biotechnological Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST). The MEGA-X (Molecular Evolutionary Genomics Analysis Version-X) constructed dendrograms from the isolates from the current study and reference strain sequences from GenBank. There were 100 replications for every bootstrap value. The pathogenic bacteria species were finally determined with the results of morphological observation, microscopic identification, and determination of pathogenic bacteria pathogenicity.

In vitro fungicide testing

Fifteen commercial chemical fungicides were considered. Before putting PDA into Petri dishes, fungicides were added in five different concentrations of the active ingredients: 10, 30, 50, 70, 100ppm. Fungicide and active components (ai) are listed in Table 1.

At the end of the trials, the percentage of mycelial growth reduction (GR%) was calculated using the formula: $GR \% = \frac{RC - RA}{RC} \times 100\%$

RC: colony radius (mm) of the control strain.

RA: colony radius (mm) of the strain in dual culture with the pesticides in amended PDA.

Table.1 Fungicides and biocontrol agents used for in vitro testing

COMMERCIAL PRODUCT	ACTIVE INGREDIENT	COMPANY
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Chemicals		
Hexaconazole	hexaconazole	Guangdong Zhongxun Agricultural Science Co., Ltd, CHINA
Xin Jun Ma Lin Gua	Moroxydine hydrochloride Otreotide acetate	Qingdao Runtian Biotechnology Co., Ltd, CHINA
Tricyclazole	Tricyclazole	Guangdong Zhongxun Agricultural Science Co., CHINA
Jia Ji Liu Junling	Thiophanate-Methyl	Guangdong Zhongxun Agricultural Science Co., Ltd, CHINA
5-Xiao Duojunling	Carbendazim Pentachloronitrobenzene	Guangdong Zhongxun Agricultural Science Co., Ltd, CHINA
Bai Junqing	Chlorothalonil	Bannong Sida (Beijing) Agro-Chemicals Co., Ltd, CHINA
Duo Junling	carbendazim	Shanghai Yilian Chemical Co., Ltd,, CHINA
Dai Sengxin	Dithane z-78	Qingdao Runtian Biotechnology Co., Ltd, CHINA
Jia Ji Tuo Bu Jing	Thiophanate-Methyl	Qingdao Runtian Biotechnology Co., Ltd, CHINA
Fu Meishuang	thiram	Beijing Green Agricultural Science and Technology Goup Co., Ltd, CHINA
Biological		
Oligosaccharins	oligosaccharins	Beijing Green Agricultural Science and Technology Goup Co., Ltd, CHINA
Streptomycin	streptomycin	Qingdao Runtian Biotechnology Co., Ltd, CHINA
Streptomycin sulfate	Streptomycin sulfate	Beijing Boatuo Technology Goup Co., Ltd, CHINA
Oxytetracycline	Oxytetracycline	Beijing Boatuo Technology Goup Co., Ltd, CHINA
Gentamycin sulfate	Gentamycin sulfate	Beijing Boatuo Technology Goup Co., Ltd, CHINA

Results

Multiple fungal strains were isolated and purified from gardenia leaf blight collected in the field. The pathogenicity was determined according

to Koch's rule, and one of them was determined to be the pathogen of the disease, which was temporarily named GD1.

Morphological characterization and identification

Morphological characteristics of the causal pathogens were studied both on the host and artificial culture medium to identify the associated pathogen, which was studied on the PDA medium. The pure culture of the GD1 initially produced white colonies (Fig. 1A), which gradually turned to light brown in 2 days and attained a growth of 9 cm in 7 days. Microscopic observations revealed that the hyphae are smooth and septate, the conidia are chain, the base is blunt and round, the other end is pointed, and there are vertical and horizontal septa. Generally, there are 1-3 transverse septa, 1-6 mediastinum, the spore size is 9.9-35.2 μm \times 5.9-12.5 μm , the average size is 18.8 μm \times 7.9 μm (Fig. 1B).

These characteristics are consistent with the morphological characteristics of *Alternaria alternata* (Fr.) Keissler.

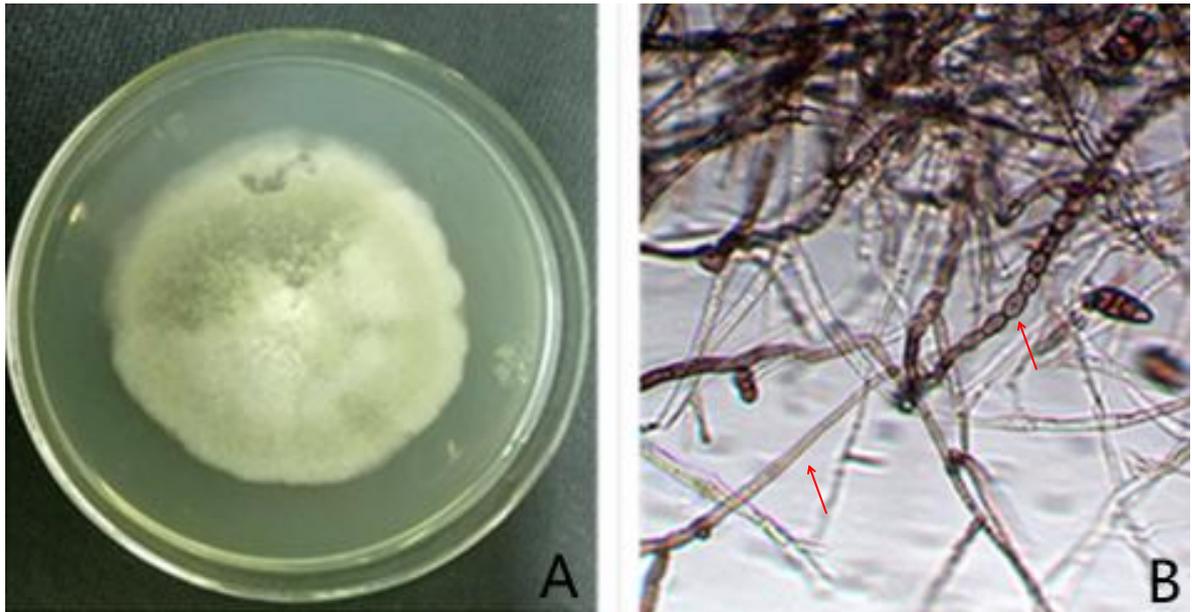


Fig1. Morpho—cultural characteristics of GD1

Pathogenicity and Symptomatology

Leaves inoculated with GD1 mycelium showed similar symptoms to naturally occurring leaf blight after two days of moisturizing at room temperature, while leaves inoculated with PDA medium showed no symptoms. The pathogens were re-isolated from the inoculated seedlings and compared with their original inoculated cultures. The re-isolated pathogens completely resembled the original inoculated fungi in their morphological, cultural and pathogenic characteristics, satisfying Koch's postulates. Pathogen inoculated plants developed initial symptoms as spotty brown discolouration of leaves. The spots were initially reddish-brown, with a diameter of 4.2-15.7mm. Subsequently, the lesion expanded and gradually turned to dark brown or black. (Figure 2A/2B.)

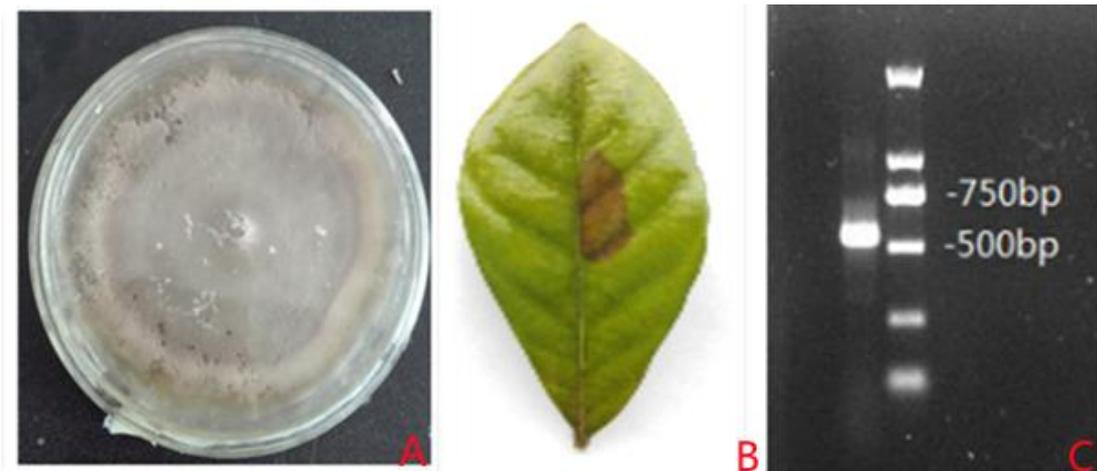


Fig2. Pathogenicity test of isolated GD1 on Gardenia leaf, and PCR product amplified from ITS Region in GD1.

Molecular Characterization and phylogeny

Pathogen template DNA and universal primers were used for rDNA-ITS sequence amplification of pathogen GD1, and a single band between 500-750bp was obtained, as shown in Figure 2C. After sequencing the PCR product and analyzing it with BLAST, our sequence showed 96%-100% sequence homology with GenBank sequences. GD1 Sequence was submitted to NCBI GenBank, and an accession number was obtained (OK041346). Phylogenetic analysis revealed that our isolates clustered along with other submitted Leaf blight isolates from GenBank. The sequences of GD1 formed the same cluster with *Alternaria sp.* (Fig. 3). Based on morphological and phylogenetic analysis, the pathogen GD1 was identified as *Alternaria alternata* (Fr.) Keissler.

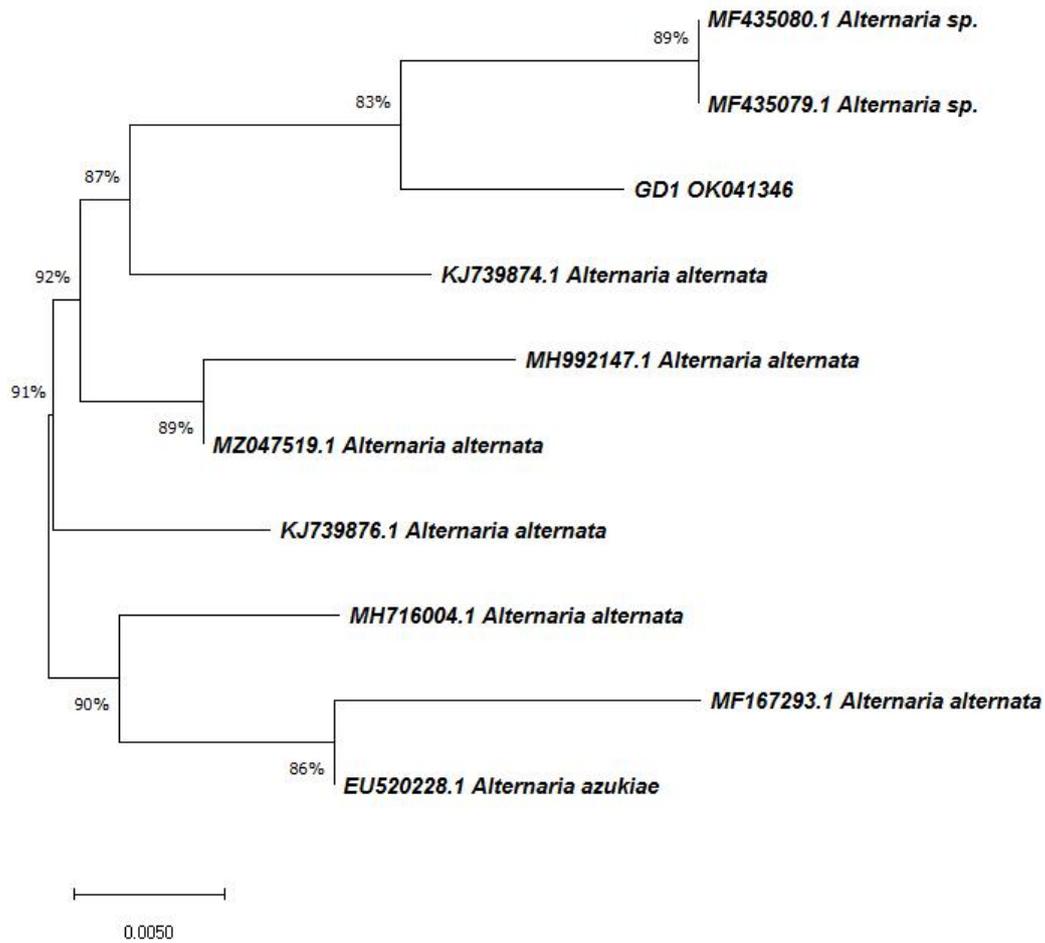


Fig3. Dendrogram generated by ITS sequence data predicting similarity

In vitro fungicide testing

Laboratory virulence tests of 15 tested fungicides against *Alternaria alternata*. showed that the inhibition effects of different fungicides were significantly different, as shown in Table 2. The best antimicrobial agents were Hexaconazole with EC₅₀ of 14.45 ppm, Chlorothalonil and Thiophanate-Methyl with EC₅₀ of 40.92 ppm, 67.92 ppm. Streptomycin(8912.51ppm), oxytetracycline(426.58ppm), Gentamycin sulfate(468.81ppm) and oligosaccharide(1196.74ppm) showed the worst

antibacterial effect, which was not suitable for the control of a large area of disease. In this experiment, all the other tested agents had different inhibitory effects on the growth of *Alternaria alternata* (Table 2).

Table 2 The toxicity comparison of 15 kinds of fungicides to *Alternaria alternata*.

ACTIVE INGREDIENT	Concentration (ppm)	GR (%)	EC ₅₀ (ppm)
Thiram	10	1.03	67.92
	30	15.94	
	50	34.96	
	70	49.61	
	100	69.73	
	20	25.08	
Thiophanate-Methyl	40	52.77	47.53
	80	65.23	
	120	71.48	
	200	76.67	
	1	9.87	
	10	32.46	
Chlorothalonil	50	48.34	40.92
	100	61.55	
	150	70.69	
	10	6.80	
Dithane z-78	100	38.88	73.52
	300	90.73	
	400	94.85	
	500	94.90	
	100	0.29	
Thiophanate-Methyl	300	18.98	714.49
	500	37.75	
	700	49.43	
	1000	57.84	
Moroxydine hydrochloride Otreotide acetate	50	11.31	311.17
	100	21.84	
	150	28.35	
	200	38.93	
	250	45.79	

	100	5.29	
	300	29.48	
carbendazim	500	47.74	481.95
	700	64.35	
	1000	80.94	
	50	19.44	
	100	28.79	
Tricyclazole	150	40.09	151.36
	200	57.83	
	250	78.52	
	100	0.93	
Carbendazim	300	23.48	
Pentachloronitrobenzene	500	40.29	554.63
	700	61.84	
	1000	79.75	
	1	18.09	
	10	30.85	
Hexaconazole	30	56.38	14.45
	50	75.89	
	70	77.48	
	10	0.35	
	100	8.69	
Oxytetracycline	500	42.02	426.58
	1000	83.87	
	1500	84.57	
	1	4.64	
	10	31.25	
Streptomycin sulfate	100	49.82	216.82
	1000	52.86	
	1500	54.46	
	100	8.37	
	200	12.39	
Gentamycin sulfate	500	38.43	468.81
	1000	59.58	
	1500	98.29	
	200	15.96	
	500	27.30	
Oligosaccharins	1000	39.46	1196.74
	1500	52.94	
	2000	69.33	

	200	1.43	
	500	5.24	
Streptomycin	1000	9.67	8912.51
	1500	15.55	
	2000	19.81	

Discussion

Gardenia fruits are rich in natural pigments and geniposide, usually used as medicinal materials and natural dyes. And gardenia flowers smell fragrant, can be used as a landscaping plant. *Alternaria alternata* has been reported to cause leaf spot on *Rumex dentatus* in Pakistan (Siddiqui et al. 2009) and leaf blight of tomato reported from parts of Pakistan (Akhtar et al. 2010) and leaf spot on *Yucca gloriosa* in China. But *Alternaria alternata* has not been yet reported to cause gardenia leaf blight in Jiangxi province.

At present, the main measures of plant disease control still rely on pharmaceutical control. In the process of pesticide use, the effects of pesticide economy, safety and efficiency should be considered comprehensively to maximize the effectiveness of pesticides and minimize adverse factors. Comparing the bacteriostatic effects of 5 biological agents and 10 chemical agents on pathogenic bacteria, it was found that the control effect of biological agents was generally lower than that of chemical agents. The EC_{50} of streptomycin, oxytetracycline and oligosaccharide were all above 400ppm, which were not suitable for

the control of large area diseases. The main characteristics of biopesticides are environmental protection, low toxicity and low pesticide residue rate. Chemical pesticides have better effects but are not conducive to environmental protection, and the pesticide residue rate is high. Therefore, multiple aspects need to be considered comprehensively in the choice of pesticide application. Plant growth promoting rhizobacteria is an effective and eco-sustainable solution to protect crops against phytopathogens. *Bacillus safensis* STJP (NAIMCC-B-02323) from the rhizospheric soil of *Stevia rebaudiana* showed strong biocontrol activity against phytopathogen, *Alternaria alternata* (Prakash et al. 2021). As a biopesticide, biological control bacteria have the advantages of environmental protection and sustainability, and may become the next hot spot of plant protection research.

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Credit authorship contribution statement

Anna Zhao & Guangming Luo Conceptualization, Methodology, Investigation, Validation, Supervision, Funding acquisition, Writing — original draft. Yuhong Gong, Yangjin Luo, and Dandan Song: Data curation, Formal analysis, Visualization. Hongdong Xia: Methodology, Investigation, Writing—review & editing.

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Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of competing interest The authors declare that they have no conflict of interest.

Declaration of Plant samples collection Our plant samples were collected from gardenia planting base. The samples are not wild plants and have been approved by the site owner. We comply with the IUCN

Policy Statement On Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora. We strict compliance with relevant institutions, national and international guidelines and legislation.

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