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Research Article

Identification of the Pathogens and Laboratory Bioactivity Determination of the Rot Disease of Kiwifruit (*Actinidia* spp.)

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Kiwifruit is an important economic crop in the world today with a high nutritional value. It can cause huge damage by causing kiwifruit rot disease; however, at present, the control methods for this disease are limited. In this study, the rotten fruits of kiwifruit (Cultivar "Jinyan") were collected from Pujiang city (Sichuan province), Xixia city, (Henan province), Zhouzhi (Shaanxi province), Meixian city (Shaanxi province), and Bijie (Guizhou province), China, and the pathogenic fungi were identified by isolation and purification, pathogenicity test, morphological characteristics, and analysis of ribosomal DNA internal transcribed spacer (rDNA-*ITS*) sequences. The results showed that the pathogenic fungi of kiwifruit rot disease were *Botryosphaeria dothidea* and *Dothiorella gregaria*. Meanwhile, the in vitro antifungal activity of 11 kinds of fungicides and 5 kinds of plant essential oils against *B. dothidea* and *D. gregaria* were determined and the results showed that all the tested fungicides and plant essential oils had a certain inhibitory effect on *B. dothidea* and *D. gregaria*. Among them, propiconazole had the best inhibitory effect on *B. dothidea* with an EC_{50} value of 4.10 mg/L, and quinolinone had the best inhibitory effect on *D. gregaria* with the EC_{50} value of 10.05 mg/L. Moreover, the pesticides and essential oils have practical application values for prevention and treatment of fruit rot diseases pathogens.

1. Introduction

Kiwifruit (*Actinidia* spp.) is rich in nutrients [1, 2], and it is one of the wild fruit trees that have been domesticated and cultivated 100 years ago [3–5]. The first record of it seem appears to be in the Book of "Shijing" before AD 1000–500 [1], while the definitive first record is in a poem written by Censen. However, the taxonomic studies, that is, to distinguish between varieties and species were still in 1984 [6]. Although it is an indigenous fruit in China [7, 8], its large-scale cultivation only started in 1978 [5], while the commercial cultivation in New Zealand began in 1930 [9]. However, because of its origin, even if it started late, China is still the largest producer of kiwifruit in the world along with New Zealand and Italy [10], and China is also the region with the highest kiwifruit diversity [11]. According to statistics, there are more than 60 species of *Actinidia* spp. in the world, with China as the center, the distribution involves latitude 500N to the equator, from cold temperate or arctic to tropical and many other countries [11–14]. As of 2019, a total of 23 countries in the world are planting kiwifruit, with a total harvest area of about 270,000 hm^2, and accounting for 67.92% (182,000 hm^2) in China [10]. In China, 40% of the kiwifruit grown in China is green pulp, followed by yellow pulp (30%) and red pulp (30%). The yellow pulp kiwifruit is mainly "Jinyan," "Jintao," "Jinyuan," "Huayou," "Jinmei," etc. [10]. "Jinyan" kiwifruit was crossed with *Actinidia chinensis* as the female parent and *Actinidia eriantha* as the male parent by the Wuhan Botanical Garden of the Chinese Academy of Sciences in 1984 [15]. It is a kind of the kiwifruit with the characteristics of high yield, beautiful and tidy fruit, smooth skin, less hairy, high content of ascorbic acid in fruit, good quality, and good storage resistance [16, 17]. It has been promoted and planted in various regions of China, including Yunnan [17], Jiangxi [16], Sichuan [18], and Guizhou [19].

The fruit rot disease can harm many fruits, such as, apple [20], sweet pepper [21], watermelon [22], areca nut [23], tomato [24], etc. It is also one of the main diseases of kiwifruit after the near-ripening stage. The damaged kiwifruit will form lesions during severe periods and emit an alcohol smell, making it inedible. The fungi of *Botryosphaeraceae* (Ascomycota: *Dothideomycetes*) degrade and passivate pollutants are a type of fungi with great potential in environmental remediation [25, 26]. But at present, its harms outweigh its benefits. This type of fungus generally parasitizes or grows in the fruits, roots, stems, and leaves of plants, causing a variety of plant diseases [27–29]. *Botryosphaeria dothidea* belong to *Botryosphaeraceae*. It is currently recognized by domestic and foreign scholars as the main pathogen causing the kiwifruit fruit rot disease [30, 31]. It is distributed in many countries and regions: New Zealand [32–34], Iran [35], Japan [36], Chile [37], Italy [38], United States [39], South Korea [40, 41], and China [42–45]. In addition to infecting kiwifruit and causing fruit rot disease, this fungus can also cause other diseases [46–49]. With the increasingly serious damage of the fruit rot disease, the prevention and control of fruit rot disease has gradually attracted the attention of the world. For example: 11% metalaxyl-M·fludioxonil·azoxystrobin, 43% Tebuconazole, Atailin, Carbendazim, and *Bacillus polymyxa,* cuminaldehyde, geraniol, and β-citronellol have a good inhibitory effect on *B. dothidea* [50–54]. However, there are fewer repots about the rot disease in kiwifruit (Cultivar "Jinyan") [55].

In order to avoid the development of related fungal resistance and ensure the diversity of fungicides, research studies on other fungicides for fruit rot disease pathogens and new fungicides of botanical origin are necessary. Therefore, in this study, the rotten fruits of kiwifruit (Cultivar "Jinyan") were collected and the pathogenic fungi were identified. Meanwhile, the in vitro antifungal activity of 11 kinds of fungicides and 5 kinds of plant essential oils against *B. dothidea* and *D. gregaria* was determined.

2. Materials and Methods

2.1. Pathogen Identification and Pathogenicity Test. The rotten kiwifruit was collected from Pujiang city (Sichuan province), Xixia city, (Henan province), Zhouzhi (Shaanxi province), Meixian city (Shaanxi province), and Bijie (Guizhou province), China (Figure 1), and packaged in a clean Ziplock bag, then was taken back to the Guizhou Engineering Research Center for Mountain Featured Fruits and Products, Guizhou Light Industry Technical College, and stored at 4°C.

The kiwifruits are first rinsed with tap water and ultrapure water 3 times, respectively, and then ventilated for 30 min to dry. The infected tissues ($1 \times 1 \times 0.5$ cm size) were soaked in 75% alcohol for about 30 s, rinsed with sterile water 3 s, and then the tissues were plated on the PDA plates. After that, the PDA plates were maintained in a constant temperature incubator at 26°C without light. After 3 days, all the strains were cultured on the new PDA plates using a single spore technique to ensure purity. Finally, the purified strains were stored at 4°C for further use.

Pathogenicity determination of pathogenic fungus was performed according to Koch's law. The healthy and near-ripe kiwifruits (Cultivar "Jinyan") were soaked in 75% alcohol for 60 s, washed with sterile water 2-3 times, and then placed on the filter papers for 15 s to absorb moisture. A sterile inoculation needle was used to pierce the middle epidermis of the cleaned kiwifruits to form a 0.2 mm wound, and a 0.5 cm sterile punch was used to make a fungus cake, and the mycelial surface of the fungus cake was attached to the wound. The sterile distilled water served as a negative control. Each treatment was repeated three times. After that, the kiwifruits were incubated in a 26°C constant temperature incubator with a humidity of 60% and a photoperiod of 12L : 12D. The surface of healthy and nearly mature kiwifruits inoculated with sterile water served as a control. After 7 days of inoculation, some symptoms have been observed on the surface. The causal fungus in the infected kiwifruit surface was re-isolated on the PDA plate as described above. The characteristics of the re-isolated fungus was used to compare with its original culture. The pathogenic fungi separation rate and the disease severity index (DSI) of Koch's test were calculated according to the following formulas. In the formulas, 0, 1, 3, 5, and 7 represent different disease levels (0: no disease; 1 : 0% < disease plaque size < 10%; 3 : 10% < disease plaque size < 25%; 5 : 25% < disease plaque size < 50%; 7 : 50% < disease plaque size), and A, B, C, D, and E represent the number of seedlings within each disease severity levels [56].

$$\begin{aligned}&\text{Separation rate (\%)}\\&\quad=\frac{\text{Number of separate pathogenic fungi kiwifruits}}{\text{Total number of test kiwifruits}}\times 100,\\&\text{DSI}\quad=\left(\frac{(0A+1B+3C+5D+7E)}{4(A+B+C+D+E)}\right)\times 100.\end{aligned}\tag{1}$$

2.2. Morphological and Molecular Identification. Individual colony was inoculated on the PDA plate and maintained in a constant temperature incubator at 26°C without light for 8 days. Then, the morphology was identified by both eye and an inverted microscopy (ECLIPSE Ni-E, Nikon Corporation, Japan).

The fungus DNA extraction was performed using the DP336 kit (Beijing Tsingke Biotechnology Co., Ltd. Chengdu Branch (BT)), and the steps were referred to the kit's instructions. The extracted DNA is amplified by PCR reaction to obtain the target gene fragment. The reaction system: 12.5 μL 2xEs Taq Mix (BT), 1 μL forward primer (ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′), 1 μL reward primer (ITS4: 5′-TCCTCCGCTTATTGATATGC-3′) [57], 1 μL DNA, and 9.5 μL ddH_2O. Reaction conditions were as follows: pre-denaturation at 94°C for 5 min, 35 cycles (denaturation at 94°C for 30 s, annealing at 56°C for 1 min, extension at 72°C for 1 min), extension at 72°C for 7 min, and

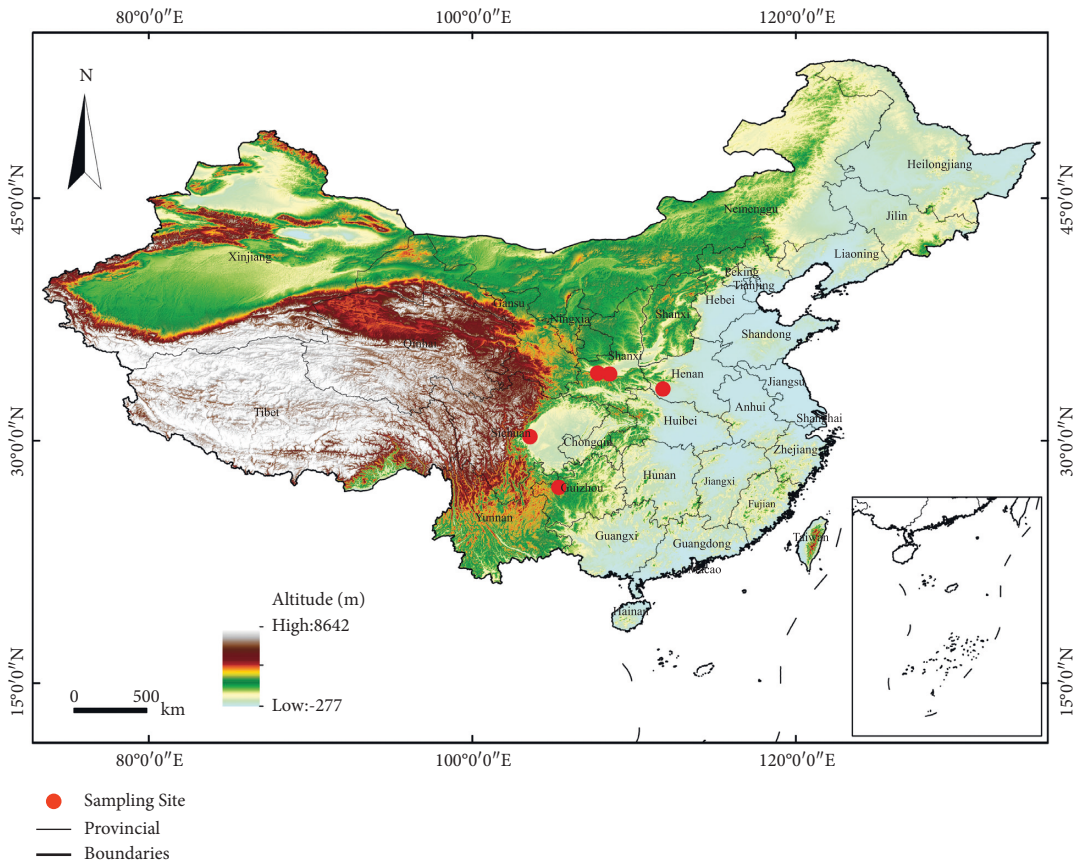

Figure 1: Distribution map of kiwifruit sample collection points.

stored at 4°C. The amplified products were sequenced by Applied Biosystems (3730XL) equipment.Viewing and calibration of sequences were performed in BioEdit (version 7.0.9.0) to obtain high-quality sequences. The obtained high-quality sequences were aligned and identified in the NCBI (https://www.ncbi.nlm.nih.gov/). Moreover, the sequences were upload to the Genbank database to obtain the accession numbers of ON566021 and ON566022.

2.3. Phylogenetic Tree Construction. Referring to the research of Zheng et al. [47], *Tiarosporella graminis* (Genbank: KC769962.1) was selected as the outgroup comparison. High-quality sequences were aligned by MAFFT (version 7.149b) [58]. The aligned sequences were edited in gBlocks (version 0.91b) software to obtain conserved sequences [59]. The ML tree of all sequences was reconstructed in MEGA (version 7.0.26), and the base substitution model used GTR + G + I clade support was estimated by bootstrap analyses with 1,000 replicates [60].

2.4. In Vitro Antifungal Activity Test. In this study, 11 kinds of fungicides and 5 kinds of plant essential oils (Table 1) against *B. dothidea* and *D. gregaria* were determined according to the reported method [53]. The inhibition rates I (%) are calculated after 7 days by the following formula, where C (cm) and T (cm) represent the fungi diameters of the CK and treated PDA plates, respectively. Meanwhile, the EC_{50} values of 11 kinds of fungicides and 5 kinds of plant essential oils against *B. dothidea* and *D. gregaria* were calculated with SPSS 19.0 software.

$$\text{Inhibition Rate } I\,(\%) = \left(\frac{(C - T)}{C}\right) \times 100. \tag{2}$$

3. Result

3.1. Pathogenicity Determination. Figure 2 shows that the strain F1 and strain F4 can cause kiwifruit rot disease. Moreover, 7 days after the pathogenicity test, the pathogenic rates of F1 and F4 strains are both 100%, and the DSI is 10.7% (Table 2). Therefore, the F1 and F4 strains are the pathogens of kiwifruit rot disease. The diseased kiwifruit was re-isolated, and the strains with the same morphological characteristics as the original inoculated strains were obtained, which met the requirements of Koch's law.

3.2. Morphological Identification and Sequence Identification. Figure 3(a) shows that the hyphae of strain F1 were initially transparent and grew in an irregular circular shape with a fast growth rate. The color gradually changed to white and off-white with time and began to appear light gray on the 3rd day (Figures 3(a) and 3(c)). In the later stage of observation, the color of the hyphae was dark green and the hyphae branched more and intertwined with each other

Table 1: List of 11 kinds of fungicides and 5 kinds of plant essential oils.

Names	Source
33.5% quinolinone SC	Shanghai hulian biological pharmaceutical co., Ltd
250 g/L propiconazole EC	Shandong xinxing pesticide co. Ltd
25% myclobutanil EC	Zhejiang yifan biotechnology group co., Ltd
25% bromothalonil WP	Jiangsu tuoqiu agrochemical co. Ltd
3% zhongshengmycin WP	Fujian kaili bio-product co., Ltd
80% ethylicin EC	Henan kebang chemical co, Ltd
100 g/L cyazofamid SC	Henan guangnong pesticide factory
10% polyoxin WP	Shanghai hulian biological pharmaceutical co., Ltd
1% osthol AP	Inner Mongolia qingyuanbao biological technology co., Ltd
0.3% eugenol AP	Jiangsu nantong shenyu green medicine co., Ltd
20% triazolone EC	Chongqing yiershuangfeng technology co., Ltd
75% chlorothalonil WP	Shandong luobang biopesticides co. Ltd
66% dithianon WG	Jiangxi heyi chemical co., Ltd
Patchouli essential oil	Beijing maosi trading co., Ltd
Ylang-ylang essential oil	Beijing maosi trading co., Ltd
Garlic essential oil	Beijing maosi trading co., Ltd
Cedarwood essential oil	Beijing maosi trading co., Ltd

Figure 2: The symptoms of F1 and F4 strains in pathogenicity test. (a) and (d): Natural occurrence characteristics of kiwifruit fruit rot disease, (b) and (e): pathogenicity test CK group, (c) and (f): pathogenicity test characteristics; (a)–(c) F1 strain. (d)–(f) F4 strain. Scale bar: 10 mm.

(Figures 3(a) and 3(c)). Its morphology was consistent with the descriptions of Liang and Ferguson [6] and He et al. [61].

Figure 3(d) shows that the hyphae of strain F4 were felt-like and the hyphae were gray in the early stage of growth. The hyphae were pale yellow-green and the center was dark gray on the 4th day. On the 8th day, the diameter of the fungus covered the petri dish (diameter = 90 mm) (Figure 3(e)). Figure 3(d) shows that the hyphae had more branches, thinner hyphae, and vigorous hyphae growth. As the growth days increased, the hyphae were dark gray in the

Table 2: Separation rate of pathogenic fungi of kiwifruit fruit rot and pathogenic rate and DSI of pathogenicity test.

Strain	Separation rate (%)	Pathogenic rate (%)	DSI (%)
F1	50	100	10.7
F4	5	100	10.7

Data statistics after seven days of pathogenicity test.

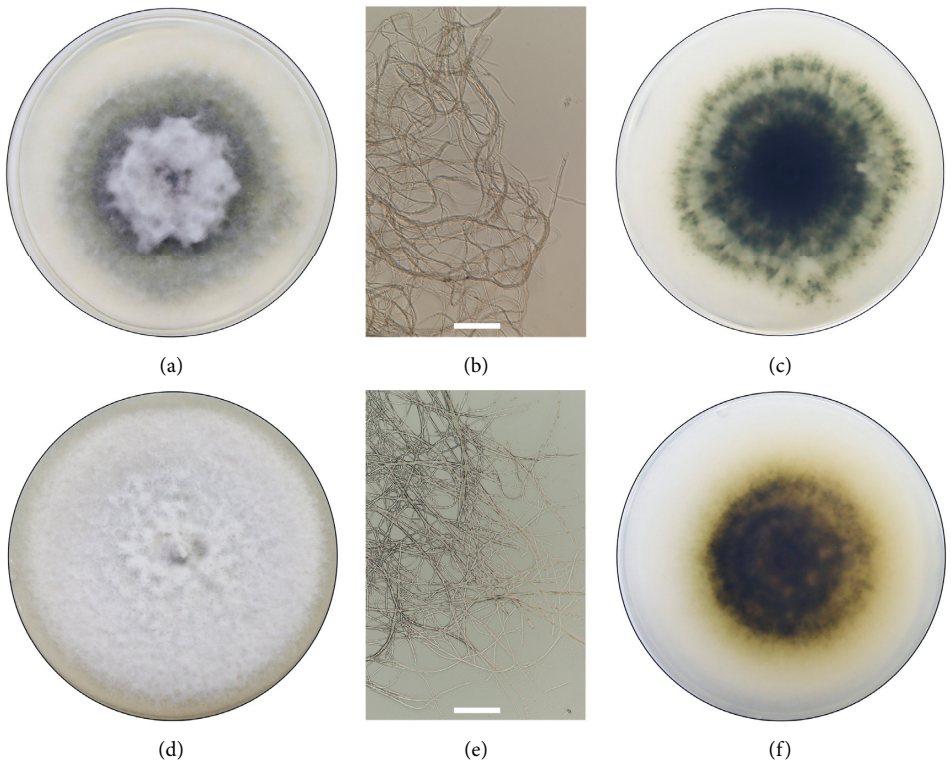

Figure 3: Morphological characteristics of pathogenic fungus of kiwifruit fruit rot disease. (a) and (d) observe surface of colony (front), (c) and (f) observe surface of colony (back), (b) and (e) mycelial morphology. (a)–(c) F1 strain. (d)–(f) F4 strain. Scale bar: 100 nm.

middle, white at the edges, and black at the bottom of the medium (Figure 3(f)). The morphology is basically consistent with the descriptions of Saccardo [62] and Zhao and Huang [63].

The phylogenetic tree was constructed using the MEGA 7.0 based on the *ITS* sequence and the results shown in Figure 4. As shown in Figure 4, the F1 and F4 strains were classified as *Botryosphaeria dothidea* and *Dothiorella gregaria*, respectively.

3.3. In Vitro Antifungal Activity. It can be seen from Table 3, the test fungicides and plant essential oils revealed different degrees of inhibition on the growth of *B. dothidea* and *D. gregaria*. Especially, quinolinone showed the best inhibitory effect against *D. gregaria* with the EC_{50} value of 10.05 mg/L; meanwhile, propiconazole had an EC_{50} value of 4.10 mg/L against *B. dothidea*, which was even better than those of other fungicides and plant essential oils.

4. Discussion

Since the first report of kiwifruit fruit rot in 1985 [64], it has been studied by various scholars one after another. At present, a variety of pathogens have been found, such as *B. dothidea*, *Botryotinia fuckeliana*, *Alternaria alternata*, *Cylindrocarpon candidum*, etc [33, 43, 64, 65]. However, *D. gregaria* is the first report that can cause fruit rot in kiwifruit. It was previously reported as the causative agent of poplar canker [66, 67], jujube fruit shrink disease [68, 69], jujube fruit black rot disease [70], cedar dieback disease [71, 72], citrus [73]. In previous studies, there are fewer control methods for this fungus: the combination of 20.67% Wanxing EC + 68.75% Yibao dispersible granules + 72% streptomycin soluble powder had a good control effect on the fungus, and the field control effect on jujube shrinkage fruit disease reached 86.9% [68]. Among the substances screened in this study, quinolinone has the lowest EC_{50} value, reaching 10.05 mg/L, such that the agent should be able to achieve a good effect in the field control of rhesus monkey fruit rot.

Among the agents we screened against *B. dothidea*, propiconazole has the best effect, and its EC_{50} reached 4.10 mg/L after 7 days, so propiconazole is recommended as an effective control agent for kiwifruit fruit rot caused by *B. dothidea*. Besides, the antifungal effect of monoterpenes on *B. dothidea* showed that cuminaldehyde had the best

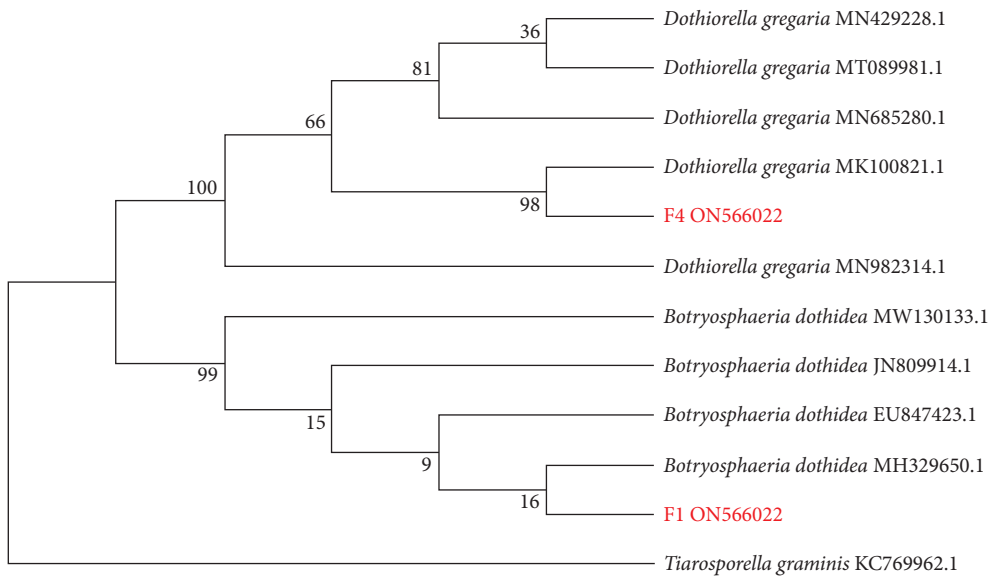

Figure 4: ML Phylogenetic tree based on *ITS*-rDNA gene sequence of kiwifruit fruit rot disease pathogen. Posterior probabilities from 1,000 bootstraps inferences are given node dates. The out group is *Tiarosporella graminis*.

Table 3: The EC_{50} values of the test fungicides and plant essential oils against *Botryosphaeria dothidea* and *Dothiorella gregaria*.

Treatment	*Dothiorella gregaria*		*Botryosphaeria dothidea*	
	Regression equation	EC_{50} (mg/L)	Regression equation	EC_{50} (mg/L)
Quinolinone	$Y = 3.9521 + 1.0456\ X$	10.05	$Y = 2.8548 + 0.9647\ X$	167.40
Propiconazole	$Y = 3.8706 + 1.1022\ X$	10.58	$Y = 4.4247 + 0.9394\ X$	4.10
Cyazofamid	$Y = 3.7769 + 0.9738\ X$	18.03	$Y = 3.1692 + 1.0047\ X$	66.40
Myclobutanil	$Y = 3.6815 + 1.0232\ X$	19.44	$Y = 3.9907 + 1.1013\ X$	8.25
Bromothalonil	$Y = 3.4510 + 0.9517\ X$	42.43	$Y = 3.6706 + 1.0776\ X$	17.12
Eugenol	$Y = 2.9490 + 1.2041\ X$	50.51	$Y = 3.2386 + 1.1044\ X$	39.34
Zhongshengmycin	$Y = 2.8882 + 1.0207\ X$	117.24	$Y = 3.5394 + 1.0988\ X$	21.35
Osthol	$Y = 2.5885 + 1.1629\ X$	118.52	$Y = 2.7987 + 1.4142\ X$	36.02
Polyoxin	$Y = 2.7142 + 1.0970\ X$	121.27	$Y = 2.4053 + 1.2884\ X$	103.25
Ethylicin	$Y = 2.5530 + 1.1350\ X$	143.22	$Y = 2.1970 + 1.4658\ X$	81.72
Chlorothalonil	$Y = 2.7630 + 0.8777\ X$	353.73	$Y = 3.5119 + 0.8690\ X$	51.57
Triazolone	$Y = 1.9850 + 1.1435\ X$	433.08	$Y = 2.8361 + 1.0914\ X$	96.10
Dithianon	$Y = 1.4391 + 1.2688\ X$	640.33	$Y = 1.1043 + 1.4664\ X$	453.47
Patchouli essential oil	$Y = 2.6529 + 1.1907\ X$	93.56	$Y = 2.7897 + 1.0509\ X$	126.84
Cedarwood essential oil	$Y = 2.7182 + 1.1448\ X$	98.43	$Y = 2.7233 + 1.0809\ X$	127.73
Garlic essential oil	$Y = 2.4979 + 1.2248\ X$	110.37	$Y = 3.0058 + 1.0010\ X$	98.21
Ylang-ylang essential oil	$Y = 2.3641 + 1.2185\ X$	145.65	$Y = 2.9745 + 1.0192\ X$	97.14

effect with the EC_{50} of 105.2 mg/L [54]; however, the bioactivity was lower than that of ylang-ylang essential oil reported in our present study (EC_{50} = 97.14 mg/L). Although there are many studies on plant essential oils or their volatile substances to control pests [74–78], the current application needs to be accelerated to provide new pesticides for comprehensive pest control.

5. Conclusion

In conclusion, our results showed that *B. dothidea* and *D. gregaria* were the pathogenic fungi of kiwifruit (Cultivar "Jinyan") rot disease in China. Meanwhile, quinolone and propiconazole revealed the best inhibitory effect on *D. gregaria* and *B. dothidea*, respectively. Our study could provide a theoretical basis for the effective control method of kiwifruit rot disease in China.

Data Availability

All data included in this study are available upon request by contacting the corresponding author.

Disclosure

This research is the achievement of Guizhou Province Academic Pioneer and Academic Pioneer Construction.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Tao Wang and Yanling Ren contributed equally to this article.

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