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Resistance analysis of grape *Botrytis cinerea* based on PCR and sequencing technology

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ABSTRACT

Resistance of grape *Botrytis cinerea* was analyzed based on PCR and sequencing technology, which provided a reasonable basis for guiding the species and usage of grape *Botrytis cinerea*. 104 monospora strains of grape *B. cinerea* were collected from the main grape-producing areas of China. After isolation and purification, the grape *B. cinerea* DNA was extracted, and PCR primers were designed according to the resistance mechanism of pyrimethene to pyrimethamines, pyridamines and diformolines. Sequence analysis showed that the resistance frequency of *B. cinerea* to pyrimidine was 22.22%-62.5%, the high-resistant strain frequency was 44.23%, and the mid-temperature zone was 62.5%. The total resistance rate of *B. cinerea* to pyrimidine was 42.60%. The resistance rate of *B. cinerea* to pyrimidine was 42.60%. Resistance frequencies of grape gray mold strains were significantly different. The resistance frequencies of grape gray mold strains to pyridimide were significant. The highest resistance frequencies of grape gray mold strains were 53.42% in the cool temperature zone. Only 3.85% of grape gray mold strains showed resistance to pyridimide. Therefore, the resistance of Grape *B. cinerea* to pyrimidine and pyrimidine is relatively common, and the resistance to enoylmorpholine is still in the initial stage.

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Introduction

Grape is the second most productive fruit in the world. China is the country with the largest grape production in the world. The planting area ranks second in the world, with 100 varieties (1). Grape diseases are one of the important factors in Weili and the grape industry. According to the survey, there are more than 40 kinds of diseases harming grape, such as downy mildew, gray mildew and powdery mildew, among which gray mildew is one of the main diseases affecting grape production (2). As long as the grape is in the growth stage, flowering stage and fruiting stage, the grape will be destroyed on a large scale, directly or indirectly leading to the reduction of grape yield under the conditions of suitable growth of gray mold. Gray mold is a destructive disease on grape trees. It causes about 20% - 30% direct loss of grape yield every year and can reach 50% of the total yield in serious cases. Grape production is seriously affected by grape mold, which restricts the development of the grape economy (3).

Botrytis cinerea is a widely parasitic saprophytic fungus. Mycelium brown, septate, conidial peduncle slender, gray-black, size of (960-1200)μm×(16-22)μm, shape like a tree, with branches, in the top of the mycelium cells expand like a sphere, around which there are some pedicels growing above, pedicels can produce many conidia similar to the round, size of [8-14)] μm×[6-9]μm, sclerotia black or brown, in a circular shape (4). Around the sclerotia of *B. cinerea* is ripe silk tissue, and inside the sclerotia is pseudo-thin tissue. *Staphylococcus fusiformis* belongs to ascomycetes; *Staphylococcus fusiformis* belongs to asexual fungi; and *Staphylococcus fusiformis* belongs to asexual fungi and resembles grape spike. *B. cinerea* has strong saprophylaxis, a wide host range and can infect each other among different host plants. At present, chemical control is the main method to control grape gray mold. The pollution-free control technologies such as disease-resistant varieties, agricultural control, biological control, physical control, natural products and safety compound control are selected as follows. At present, chemical control is the most effective means. With the wide use of

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chemical agents, *B. cinerea* has the characteristics of frequent genetic variation, high suitability and fast growth. With the continuous and wide use of chemical agents, it is easy to make *B. cinerea* resistant to fungicides (5). Therefore, the analysis of resistance of *B. cinerea* is very important.

Zhou Mingguo and others first detected *B. cinerea* B1 resistant to carbendazim in Nanjing, and its resistance gradually increased with the increasing range and dosage of the fungicides. However, benzimidazole fungicides still account for a certain proportion in the control of *B. cinerea*. Xiaoting et al. showed that mutation of histidine to leucine (H272L) at site 272 of Sdh B subunit could induce drug resistance of the strain, but this type was rarely found. In addition, Xu Yuan et al. showed that the mutation of proline to phenylalanine (P225F), threonine (P225T) or leucine (P225L) at position 225 of Sdh B subunit and aspartic acid at position 230 to isoleucine (N230I) were the causes of resistance.

Traditional detection methods for resistance to fungicides of *B. cinerea* are the mycelial growth rate method and spore germination method (6). However, these two traditional detection methods have some disadvantages, such as complicated operation, large test workload, long cycle and poor sensitivity. In addition, the traditional method for testing the resistance of *B. cinerea* strains is easily interfered with by many factors, such as the solubility of fungicides, the inhibition of organic solvents on *B. cinerea* itself, and so on (7). Therefore, traditional detection methods are gradually being replaced by molecular methods. At present, molecular detection technology has been used in various fields and is favored by the majority of scientific researchers because of its high sensitivity and easy operation. At present, with the clarity of the resistance mechanism of some commonly used fungicides, molecular detection techniques have been widely used in resistance detection, such as fluorescence quantitative PCR, ARMS-PCR, PCR-RFLP, PIRA-PCR, high-throughput sequencing, etc.

High throughput sequencing technology in molecular detection technology can sequence hundreds of thousands to millions of DNA molecules in parallel at one time. It can deeply study the genome structure, function, expression and regulation of various species. High throughput sequencing technology has the advantages of high sensitivity, high throughput, low cost, fast detection speed and wide coverage (8). Transcriptions and genomes of a species can be comprehensively and thoroughly studied, so they are widely referred to as deep sequencing. The principle is to connect genomic DNA fragments to universal joints, amplify the genomic DNA by different amplification methods, and obtain a large number of single-molecule polyclonal sequence products. Then a series of primer hybridization and enzymatic extension reactions are used to sequence the products in parallel, and a large number of sequencing data are obtained through biotechnology. Complete DNA sequence information can be obtained by analysis.

Therefore, in order to fully understand the resistance of *B. cinerea*, PRC and high throughput sequencing techniques were used to analyze the resistance of *B. cinerea* to pyrimidine, pyrimidine and enoylmorpholine.

Materials and methods

Test material

Source of strain

The experiment was completed in the Institute of Plant Protection and Environmental Protection, Beijing Academy of Agricultural and Forestry Sciences from 2010 to 2011. A total of 104 strains of Grape *B. cinerea* were isolated and purified from samples collected from different climatic regions of grape cultivation in China. The strains were preserved in (9) Institute of Plant Disease Control, Beijing Academy of Agricultural and Forestry Sciences, through the inclined plane (4 ℃) and filter paper method at low temperature (-20 ℃). The strains of Grapevine *B. cinerea* tested are shown in Table 1.

Main instruments

Ultra-clean workbench, TH-250 gradient mixer, multi-sample tissue grinder (Shanghai), constant temperature magnetic stirrer, Eppendorf 5424 centrifuge (Germany), Bio-RAD (USA) Universal Hood II gel imaging system, Eppendorf Mix Mate vortex oscillator, Eppendorf (Germany) centrifuge, Lance liquid gun, instrument, Illumination incubator, cryogenic refrigerator, 4 ℃ refrigerator, high-pressure steam sterilizer, nitrogen cylinder, 5 mm perforator, scalpel drying water bath pot and other auxiliary equipment.

Table 1. Grape *B. cinerea* strains tested

Collection site	Strain number					
Cool and temperature zone	GSLZnky4-1	GSLZnky13-6	GSLZyd10-2	SXQXIj1-1	LNXCetz1-1	LNXCetz7-1
	GSLZnky5-2	GSLZnky14-1	GSLZydll-1	SXQXlj2-1	LNXCetz2-1	LNXCetz8-1
	GSLZnky6-5	GSLZyd5-2	GSTSst5-2	NXGQyjs1-1	LNXCetz3-1	LNXCjc9-1
	GSLZnky7-1	GSLZyd6-1	GSZYyx3-1	NXGQyjs2-1	LNXCetz4-1	
	GSLZnky9-1	GSLZyd7-4	GSZYyx4-1	NXGQyjs3-1	LNXCetz5-1	
	GSLZnkyll-1	GSLZyd9-3	SXQXhzs1-1	NXYNI-1	LNXCetz6-1	
Mesothermal zone	HeBCLzgz-1	SDTK6-1	SDXI3-1	SDXI10-1	SDPLscll-1	SDXX7-1
	SDTK1-1	SDTK7-1	SDXI4-1	SDPL1-1	SDLT1-1	HBCLhx2-2
	SDTK2-1	SDTK8-1	SDXI6-1	SDPL2-1	SDXX3-4	
	SDTK3-1	SDTK9-5	SDXI7-1	SDPL3-1	SDXX4-2	
	SDTK4-1	SDXL1-1	SDXI8-1	SDPL4-1	SDXX5-1	
	SDTK5-1	SDXL2-1	SDXI9-1	SDPLxsy5-1	SDXX6-1	
Warm temperature zone	TJCD1-1	BJYQzsy3-1	BJYQzsy8-3	BJYQzsy14-3	BJCP1-1	BJSYtz1-1
	TJCD2-1	BJYQzsy4-1	BJYQzsy10-1	BJYQzsy15-1	BJCP2-2	TJCD4-1
	TJCD3-1	BJYQzsy5-1	BJYQzsy11-2	BJYQzsy16-1	BJCP3-2	
	BJYQzsyl-1	BJYQzsy6-2	BJYQzsy12-1	BJYQzsy17-1	BJCP4-1	
	BJYQzsy2-1	BJYQzsy7-3	BJYQzsy13-3	BJYQzsy19-1	BJCP5-1	
Humid-hot zone	JSCZ1-1	HuBzj2-1	ZJHZyys3-1			
	HuNhhl-1	HuBzj3-1	HuNhh4-1			
	HuNhh2-1	HuBzj4-3				
	HuNhh3-1	ZJHZyys1-1				
	HuBzj1-2	ZJHZyys2-1				

Main reagents

KCl, glucose, hydrochloric acid, sodium hydroxide, L-asparagine, Tween 20, EDTA, agar, yeast extract, malt extract, ddH20, Tris, isopropanol, absolute ethanol, OMEGA company purchased E-Z96 fungal genome DNA extraction kit, Axygen AxyPrep TMDNA Gel Extraction Kit, agarose, liquid nitrogen.

Culture medium

(i) PDA medium: glucose: 20g, potato: 200g, agar: 10g, adding ddH2O to 1 L, sterilizing for 20 minutes under high-pressure steam at 121℃.

(ii) WA medium: agar: 20g, adding ddH2O to 1 L, sterilizing at 121℃ and high-pressure steam for 20 minutes.

(iii) HA medium: yeast extract: 4g, glucose: 4g, malt extract: 10g, agar: 15g, add ddH2O to 1L, and adjust PH to 5.5. Sterilize for 20 minutes at 121℃ under high-pressure steam.

The standard for classification of the resistance level of grape *B. cinerea*

The resistance level was judged by the sensitive baseline value (0.0911 μg/mL). When the EC50 value of the strain was less than 5 times of the sensitive baseline, that is, when the EC50 value of the strain was less than 0.4555 μg·mL-1, it was the sensitive strain; when the EC50 value of the strain was between 5 and 10 times of the sensitive baseline, that is, when the EC50 was between 0.4555 and 0.911μg·mL-1, it was the low-resistant strain. The EC50 value of a strain was between 10 and 40 times of the sensitive baseline, i.e. when EC50 was 0.911-644 μg·mL-1, it was a medium-sized antimicrobial strain; when the EC50 value of a strain was 40 times higher than that of the sensitive baseline, that is, when EC50 was higher than 3.644μg·mL-1, it was a high-antimicrobial strain. The frequency of susceptible and resistant strains in different regions was counted. Resistance frequency (%)= (total number of resistant strains determined) ×100%.

Test method

Extraction of DNA from grape *B. cinerea*

The genomic DNA of the *B. cinerea* strain was extracted using the OMEGA E-ZR96 Fungal DNA Kit extraction kit (D3390-02) (10). The specific method is as follows:

Scrape about 20 mg of gray mold hyphae into a 2 ml centrifuge tube, then add two glass beads and an appropriate amount of quartz sand to the tube; Put the hyphal sample into the multi-sample tissue grinder module, quickly pour liquid nitrogen to freeze it, and then insert the module into the grinder for 1 min to fully break the sample; Add 800 μL Buffer FG1 to the ground sample, place it in the Eppendorf Mix Mate vortex oscillator and vortex it to break all the tissue blocks and mix the sample thoroughly; Place the sample in a water bath at 65 ℃ for 10 min, and mix the sample upside down at least 3 times during the water bath; After removing, add 140 μL of Buffer FG2 to the tube, mix it with a vortex, and place it in a

refrigerator at -20 ℃ for 10 min to remove the polysaccharide, protein and other inhibitors in the sample solvent. After taking out the sample from the refrigerator, the sample is placed in a centrifuge and centrifuged at 12,000 rpm for 10 minutes to precipitate the debris therein; Pipette 700 μL of the supernatant into a new 1.5 ml centrifuge tube, avoid breaking the sediment or debris, then add 490 μL of isopropanol to the supernatant, vortex and mix the solution and precipitate the DNA; After taking out, place the sample symmetrically in a centrifuge and centrifuge at 12000 rpm for 2 min. Pour off the supernatant under the condition that the sediment is not poured out, and then fold the tube down on the absorbent paper for 1 min to make the residue. Add 300 μL of ddH2O at 65℃ to the centrifuge tube, vortex to re-dissolve the precipitate, then add 4 μL of RNaseA (20 mg/ml), 150 μL of Buffer FG3, 300 μL of absolute ethanol, and vortex to mix; Transfer all samples (including possible precipitation) to a white centrifuge tube with a blue adsorption column using a pipette, and centrifuge at 12000 rpm for 1 min; Throw away the white centrifuge tube containing the filtrate, then place the blue adsorption column into a new white centrifuge tube and add 700 μL of DNA wash Buffer (Note: DNA wash Buffer must be added to the absolute ethanol shake as required before use), then centrifuged at 12000 rpm for 1 min in a centrifuge and then drain the liquid from the white centrifuge tube; Re-inject the blue adsorption column into a white centrifuge tube, add another 700 μL of DNA wash Buffer, and then centrifuge at 12000 rpm for 1 min in a centrifuge, then pour off the liquid in the white centrifuge tube; Put the blue adsorption column into the white centrifuge tube, discard the white centrifuge tube after centrifugation for 2 minutes, and place the blue adsorption column on the absorbent paper for 10 minutes to evaporate all the liquid on it; Put the adsorption column into a new 1.5 ml centrifuge tube, and take 50-100 μL of Elution Buffer preheated to 65℃ and add it to the center of the adsorption column. Allow to stand at room temperature for 5 min, then put into the centrifuge at 12000 rpm for 5 min; Re-adding the liquid in the centrifuge tube to the center of the adsorption column for secondary elution; The blue adsorption column is discarded, and the extracted genome of *B. cinerea* is stored at -20℃ for use.

Establishment of high-throughput detection method for resistance of grape *B. cinerea* to three fungicides

Primer design

As shown in Table 2, primers were designed according to the resistance mechanism of grapevine to pyrimidine, pyrimidine and enoylmorpholine (11).

Table 2. Primers according to the resistance mechanism of grapevine to pyrimidine, pyrimidine and enoylmorpholine

Bactericide	Fragment	Primer Sequence F& amp; R	Length (bp)
Pyrimethanil	Tuba	CCrCCrACrCrACrTTCCCACrATCCr CrACCrCrCCrCrAAACCAACrTCrCr	233
Boscalid	SdhB	CrTATTCTCTCrCCrCATCrCTCrC CCrCAATTCrCCAAACCACrCrAT	236
Dimethomorph	mrr1	TTTCrTCTTTCrATTCCACrCrCrTCAC TACrCrCACTCTTCATTTCrTCATCr	233

Establishment of PCR system

Conventional PCR: 20 μL of PCR reaction system containing 40 ng DNA template, 0.3 μMTaq DNA polymerase, 2.5 μM 10×buffer, 2 μM 2.5×dNTP, Primer F (10 pM) and Primer R (10 pM) each 1 μL, plus ddH2O to 20 μL; PCR reaction procedure: 95℃ 5 min; 94℃ 30 s; 56℃ 30 s; 72℃ extension, 30 cycles: 72℃, 10 min; 4℃ unlimited.

Agarose gel electrophoresis of DNA

Preparation of agarose gel: appropriate amount of agarose gel, placed in a suitable cone bottle, add a certain volume of 1 × TAE buffer, heat dissolve and mix well; when the gel is cooled to 50-60℃, add the proper amount of fluorescent dye, shake it well, then pour it slowly into the gel tray that has been plugged in, and stay at room temperature for half an hour;

Sample added: agarose gel sample hole into the cathode into the electrophoresis tank, and make the TAE buffer diffuse over the glue surface; extract the 5μL PCR reaction product into the adding hole; if PCR is used without dye raw material, then we need to take the 4μL PCR reaction product and 1μL DNA sample buffer mixture and add the sample hole.

Electrophoresis: set current 200 mA, voltage 140 V, electrophoresis time depends on the DNA fragments to be detected;

Imaging: after electrophoresis, Bio-RAD (USA) Universal Hood II gel imaging system was used to observe, photograph and preserve the image.

Recovery of PCR products

Purification and recovery of DNA product fragments using the Axygen Axy Prep TM DNA Gel Extraction Kit (12), as follows:

Using a Bio-RAD (USA) Universal Hood II gel imaging system, the agarose gel containing the DNA of interest was cut under a UV lamp, and the surface liquid of the gel was blotted with a paper towel and chopped. Calculate the gel weight (pre-record the weight of the 1.5 ml centrifuge tube) as a gel volume (eg 100 mg = 100 μL volume);

Add 3 gel volumes of Buffer DE-A, mix well and heat at 75℃ (low melting point agarose gel heated at 40℃), intermittently mix (every 2-3 min until the gel block is completely melted (About 6-8 min);

Add 0.5 Buffer DE-A volume Buffer DE-B and mix well. When the isolated DNA fragment is less than 400 bp, an additional gel volume of isopropanol is required. After adding Buffer DE-B, the color of the mixture turns yellow and mix well to ensure a uniform yellow solution;

Connect the negative pressure device correctly and insert the DNA preparation tube into the socket of the vacuum device. Draw the mixture in step 3, transfer to the preparation tube, open and adjust the negative pressure to -25-30 inches of mercury, slowly sucking away the solution in the tube;

Add 500μL Buffer W1, suck in the solution;

Add 700 μL Buffer W2 and absorb the solution. Wash again with 700 μL Buffer W2 in the same manner. (Confirm that absolute ethanol has been added to the specified volume on the reagent bottle in Buffer W2 concentrate, and twice using Buffer W2 to ensure that the salt is completely removed, eliminating the impact on subsequent experiments);

Place the preparation tube in a 2 ml centrifuge tube (provided by the kit) and centrifuge at 12000 rpm for 1 min.

High throughput sequencing

First, the PCR products were examined by agarose gel electrophoresis. All the amplified products were taken out of the 5μL mixture into a 45ml centrifuge tube. After mixing, the 400μL gel was recovered. The recovery product was constructed by Axygen Axy Prep TM DNA gel extraction kit, and Illumina X-Ten system was used for PE150 sequencing (Li Lily company) (13).

Results and discussions

Analysis of resistance to pyrimidine of grape *B. cinerea*

Frequency and distribution of resistance to pyrimidine in *B. cinerea*. The EC50 values of pyrimethanil against 104 strains of *B. cinerea* from different viticultural climate zones were determined. The results are shown in Table 3. The results showed that the resistance frequency of *B. cinerea* to pyrimethanil was 22.22%-62.50%; the resistance frequency of *B. cinerea* to pyrimethanil in different grape-producing areas was different, the frequency of resistance in the warm temperature zone (Tianjin, Beijing area) was low, and the frequency of resistance was 22.22%. The highest frequency of resistance to pyrimethanil in the medium temperature region (Hebei Changli, Yantai, Shandong) was 62.50%; the resistance of *B. cinerea* to pyrimethanil The sex level is also very high, with the highest EC50 value of 78.5575 μg•mL-1, which is 1327 times higher than the minimum EC50 value of 0.0592 μg•mL-1.

Table 3. Resistance of *B. cinerea* to pyrimidine and frequency of resistance

Climatic region of grape cultivation	Number of strains (strains)	EC50 Scope(μg•mL-4)	Expressivity of resistant strains				Number of resistant strains/frequency of resistance (%)
			Sensitive	Low resistance	Moderate resistance	High resistance	
Cool and Temperature Zone	30	0.0593-28.6189	18	3	4	5	12(40.00)
Mesothermal zone	32	0.1788-72.5347	12	5	5	10	20(62.50)
Warm Temperature Zone	27	0.0884-41.6759	21	4	1	1	6(22.22)
Humid-hot zone	15	0.22-78.5576	7	0	0	8	8(53.33%)

Determination of the resistance level of *B. cinerea* to pyrimethanil. The results of the determination of the resistance level of *B. cinerea* to pyrimethanil are shown in Figure 1. The results showed that among the 104 strains tested, the resistance strain of *B. cinerea* to pyrimethanil was 46, the frequency of resistance was 44.23%, and the strain with high resistance to pyrimethanil was 24 strains. The resistance frequency has reached 23.08%, the low-resistant strain and the medium-resistant strain are 12 strains, and the

resistance frequency is 9.62%. Among the 46 resistant strains, the high-resistance and medium-resistant strains are the main ones, among which the high-resistant strains were 24, accounting for 52.17% of all resistant strains.

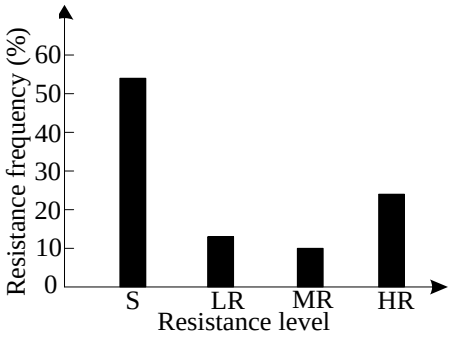

Figure 1. Resistance level and frequency of grape *B. cinerea* to pyrimidine.

Drug analysis of pyrimidinyl amide resistant to grape gray mold

Resistance of *B. cinerea* to pyridimide. Among the 104 strains, 43 strains (41.34%) were resistant strains and 61 strains (58.66%) were sensitive strains. It can be seen that a stable population of imipenem has been formed in the population of *B. cinerea*. The resistance results of *B. cinerea* to chlorpyrifos in different climate cultivation areas are shown in Figure 2. Different climatic cultivation areas have different levels of drug use, and the frequency of resistance is different. The samples collected in the cool temperature zone have the highest frequency of resistance to chlorfenapyr (53.42%), followed by the hot and humid zone (41.67%), warm temperature zone (40%), and medium temperature zone (16.67%).

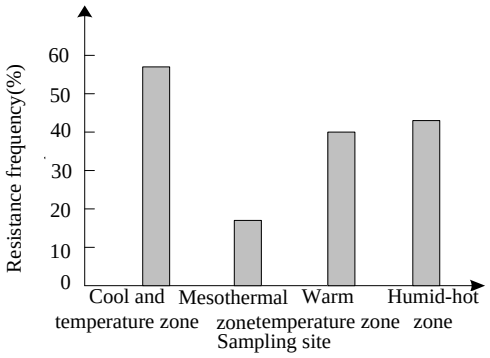

Figure 2. Resistance of *B. cinerea* to pyrimidine in different areas.

Sensitivity phenotype of *B. cinerea* to boscalid. The average EC50 of the *B. cinerea* strain EC50 was 0.0353 mg/L, and the strain sensitive strain had an EC50 value of less than 0.1000 mg/L (88.89%), as shown in Figure 3(a). The EC50 values of the resistant strains were mainly concentrated at 0.5001-2.0000 mg/L (79.17%) as shown in Figure 3(b). The sensitivity phenotype of the strain can be divided according to the EC50 ratio (RF) of the resistant strain and the susceptible strain. The frequency of distribution of the RF value of *B. cinerea* to the phytoestrazine is shown in Figure 4(a) and 4(b).

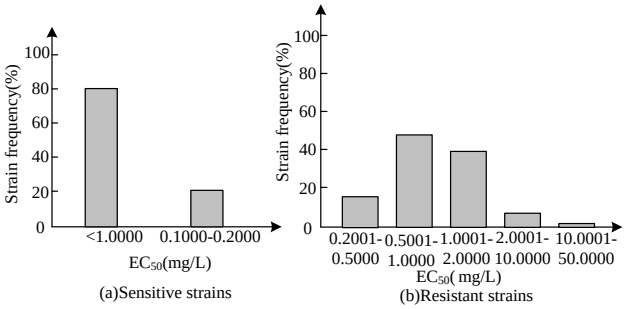

Figure 3. Distribution frequencies of EC50 of p-pyrimidinyl amide by *B. cinerea*.

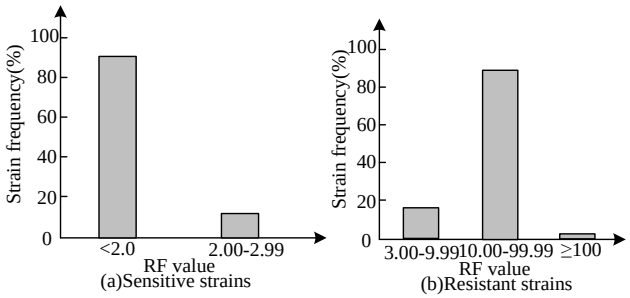

Figure 4. Distribution frequency of RF value of gray mold to phthalocyanine.

The resistant target genes (sdhB, sdhC and sdhD) of the resistant strain resistant strains were subjected to sequencing analysis, and the results are shown in Table 4. The results showed that no amino acid substitutions were found on the three subunits of sensitive strains sdhB, sdhC and sdhD. Compared with susceptible strains, codon 272 of sdhB gene of gray mould resistant strains mutated from CAC (coding histidine, H) to CGC (coding arginine, R), i.e. H272R mutation. The codon 272 of sdhB gene of a grape Gray Mould Resistant Strain mutated from CAC (coding histidine, H) to CGT (coding arginine,

R), or H272R. The codon 272 of sdhB gene of three grape gray mold resistant strains mutated from CAC (coding histidine, H) to TAC (coding tyrosine, Y), namely H272Y mutation. No resistant strains mutated on sdhC and sdhD subunits were detected.

Table 4. Detection of sdhB gene mutation and sensitivity phenotype of *B. cinerea*

Strain number	Number of strains	EC50	Sensitivity phenotype	SdhB mutation site	RF value
GSLZnky4-1, GSLZnky5-2, GSLZnky6-5, GSLZnky7-1, GSLZnky9-1, GSLZnkyll-1, GSLZnky13-6, GSLZnky14-1	8	0.0449	S	-	-
SXQXlj2-1	1	0.1317	RS	-	-
LNXCetz7-1, LNXCetz8-1, LNXCjc9-1	3	0.3031	LR	H272R	4.04-7.79
GSLZyd10-2, GSLZydll-1, GSTSst5-2, GSZYyx3-1, GSZYyx4-1	5	0.3401	LR	H272R	2.64-9.52
SDTK1-1, SDTK2-1, SDTK3-1, SDTK4-1, SDTK5-1, SDTK6-1, SDTK7-1, SDTK8-1, SDTK9-5, SDXL1-1, SDXL2-1, SDXI3-1, SDXI4-1, SDXI6-1	14	1.0943	MR	H272R	10.19-353.15
TJCD1-1, TJCD2-1, TJCD3-1, BJYQzsyl-1, BJYQzsy2-1, BJYQzsy3-1, BJYQzsy4-1, BJYQzsy5-1, BJYQzsy6-2, BJYQzsy7-3, BJYQzsy8-3, BJYQzsy10-1, BJYQzsy11-2, BJYQzsy12-1, BJYQzsy13-3, BJYQzsy14-3, BJYQzsy15-1, BJYQzsy16-1, BJYQzsy17-1, BJYQzsy19-1, BJSYtz1-1, TJCD4-1, ZJHZyys3-1, HuNhh4-1, SDPLxsy5-1	25	13221	MR	H272R	10.07-77.66
HuNhhl-1, HuNhh2-1	2	11.1708	HR	H272R	151.44-259.16
HuBzj2-1, HuBzj3-1, HuBzj4-3	3	1.4553	MR	H272Y	26.28

Note: No mutation was detected; S: sensitivity; RS: decreased sensitivity; LR: low resistance; MR: moderate resistance; HR: high resistance.

Analysis of resistance to phenylphthalmoline to grape *B. cinerea*

The DNA of 104 strains was extracted after pure culture. Tetra-primer ARMS PLR was used to detect its resistance to phenyl phthalmo line. The test results are shown in Table 5. The results showed that 100 strains contained 612 by and 238 by amplification bands, while only 4 strains contained 612 by and 378 by amplification bands.

Table 5. Resistance of *B. cinerea* to phenyl phthalmoline

Strain number	Resistance status	Strain number	Resistance status	Strain number	Resistance status	Strain number	Resistance status
GSLZnky4-1	S	GSLZnky13-6	S	GSLZyd10-2	S	SXQXIj1-1	S
GSLZnky5-2	S	GSLZnky14-1	S	GSLZydll-1	S	SXQXlj2-1	S
GSLZnky6-5	S	GSLZyd5-2	S	GSTSst5-2	S	NXGQyjs1-1	S
GSLZnky7-1	S	GSLZyd6-1	S	GSZYyx3-1	S	NXGQyjs2-1	S
GSLZnky9-1	S	GSLZyd7-4	S	GSZYyx4-1	S	NXGQyjs3-1	S
GSLZnkyll-1	S	GSLZyd9-3	S	SXQXhzs1-1	S	NXYNI-1	S
HeBCLzgz-1	S	SDTK6-1	S	SDXI3-1	S	SDXI10-1	S
SDTK1-1	S	SDTK7-1	S	SDXI4-1	R	SDPL1-1	S
SDTK2-1	S	SDTK8-1	S	SDXI6-1	S	SDPL2-1	S
SDTK3-1	S	SDTK9-5	S	SDXI7-1	S	SDPL3-1	S
SDTK4-1	S	SDXL1-1	S	SDXI8-1	S	SDPL4-1	S
SDTK5-1	S	SDXL2-1	S	SDXI9-1	S	SDPLxsy5-1	S
TJCD1-1	R	BJYQzsy3-1	S	BJYQzsy8-3	S	BJYQzsy14-3	S
TJCD2-1	S	BJYQzsy4-1	S	BJYQzsy10-1	S	BJYQzsy15-1	S
TJCD3-1	S	BJYQzsy5-1	S	BJYQzsy11-2	S	BJYQzsy16-1	S
BJYQzsyl-1	S	BJYQzsy6-2	R	BJYQzsy12-1	S	BJYQzsy17-1	S
BJYQzsy2-1	S	BJYQzsy7-3	S	BJYQzsy13-3	S	BJYQzsy19-1	S
JSCZ1-1	S	HuBzj2-1	S	ZJHZyys3-1	S	SDXX3-4	S
HuNhhl-1	S	HuBzj3-1	S	HuNhh4-1	S	SDXX4-2	S
HuNhh2-1	S	HuBzj4-3	S	LNXCetz2-1	S	SDXX5-1	S
HuNhh3-1	S	ZJHZyys1-1	S	LNXCetz3-1	S	SDXX6-1	S
HuBzj1-2	S	ZJHZyys2-1	S	LNXCetz4-1	S	BJCP1-1	S
LNXCetz7-1	S	HBCLhx2-2	S	LNXCetz5-1	S	BJCP2-2	S
LNXCetz8-1	S	BJSYtz1-1	S	LNXCetz6-1	S	BJCP3-2	R
LNXCjc9-1	S	TJCD4-1	S	SDPLscll-1	S	BJCP4-1	S
SDXX7-1	S	LNXCetz1-1	S	SDLT1-1	S	BJCP5-1	S

Note: S and R represent sensitive and resistant strains respectively.

The strains containing 612 BP and 238 BP as well as 612 BP and 378 BP were homozygotes. The strains containing 612 bp, 238 bp and 378 bp were heterozygotes. Homozygous mutants were proved to be resistant to phenylmorpholine by the traditional leaf disc method, while homozygotes and heterozygotes with sensitive alleles were not resistant to phenylmorpholine. 104 strains were homozygotes, of which 100 contained the sensitive allele of cellulose synthase 3 of *B. cinerea*. They were sensitive to phenylphthamoline and resistant to phenylphthamoline. Four strains were resistant to phenylphthamoline. This indicated that although the frequency of resistance in this area was not high, the strain of *B. cinerea* resistant to phthamoline had appeared.

Grape gray mold is an important disease in grape cultivation. It exists in almost all grape production gardens in China. Because there are fewer resistant cultivars, the control of grape gray mold mainly depends on fungicides (14) However, with the continuous and frequent use of fungicides, grapevine grey mold gradually produces resistance to fungicides, resulting in a decline in fungicide control effectiveness. Since the registration of aniline pyrimidine fungicides for the control of gray mold in the 1990s, several strains (15) resistant to aniline pyrimidine fungicides have been reported in the field. The results showed that the resistance of *B. cinerea* to pyrimidine was very common. The resistance frequency reached 44.23%. Of 46 resistant strains, 24 were moderately resistant and highly resistant, accounting for 52.17%. This was the same as the resistance of *B. cinerea* to aniline pyrimidines reported previously in China. The results showed that the resistance of *B. cinerea* to pyrimethamine was different in different climate zones of grape cultivation. The resistance frequency of *B. cinerea* to pyrimethamine was 22.22% in warm temperature zones, which was lower than that of *B. cinerea* in cool temperature zones, medium temperature zones and humid-hot zones. The resistance frequency of *B. cinerea* to pyrimethamine was 40%, 62.5% and 53.33%, respectively in these three zones, which may be related to the types of medicines used to control grape gray mold and the occurrence of grape diseases in different areas. Grape gray mold is a mild disease in warm temperate areas and is a common disease in other areas.

The resistance of Grape *B. cinerea* to pyrimidine was gradually revealed with the increase of time and frequency of use (16). The average frequency of pyrimidine-resistant strains reached 41.34%, but there were significant differences among different regions. Grape cultivation in warm and cool areas, humid and hot areas and warm temperate areas have been for a long time. Resistance to pyrimidine of *B. cinerea* population was higher, reaching 53.42%, 41.67% and 40.00% respectively. The results showed that the antipyrimidine population of *B. cinerea* in the vineyard had been stable. The sensitivity of *B. cinerea* to pyrimidine showed that the resistance of *B. cinerea* to pyrimidine was mainly moderate. Sequencing analysis of target genes (sdhB, sdhC and sdhD) of all resistant strains showed that mutations of all resistant strains occurred only at position 272 of sdhB gene, and only two mutation types of H272R and H272Y were found. These results are similar to those reported by Veloukas et al. The difference is that no mutation types such as H272L, P225F and N230I were detected in this study. In conclusion, there is a correlation between different mutation types of sdhB gene and drug sensitivity in *B. cinerea*. The strains of H272L and P225F from the sdhB gene mutation of *B. cinerea* were used to control grape fruits in vitro, and pyridimide was ineffective against them (17). In view of this, in order to provide timely information on resistance, it is necessary to continuously monitor the dynamic changes of resistance of *B. cinerea* population to pyrimidine, especially to determine whether there are other new mutation types (1).

After the emergence of strains resistant to phenylphthamolyne, the frequency of resistance will rise sharply to nearly 100% if they are used alone for several years. The control effect may be completely lost, but the frequency of resistance will decrease rapidly after two years of discontinuation. Therefore, drug resistance monitoring is an effective means to guide the rational use of such fungicides and to effectively control the resistant groups in order to prolong the service life of fungicides (18-23). Among 104 strains used in this experiment, 4 resistant strains were detected, and no heterozygous strains were found, indicating that there were strains resistant to phenylphthalmoline in this area. This result indicated

that phenylphthalmoline still had an excellent control effect on grape gray mold and was recommended as a control agent for gray mold. For the emergence of resistant strains, although the low frequency of resistance did not affect the control effect of phenylphthalmoline, it should still be paid enough attention to, and the resistance frequency should be monitored continuously. When the resistance frequency increased, timely measures should be taken to prevent the expansion of resistance genes and the further increase of resistance frequency and to reduce the losses caused by gray mold.

Conclusions

The resistance of *B. cinerea* to pyrimidine, pyrimidine and phenylphthalmoline fungicides was studied by PCR and high throughput sequencing. The results were as follows:

The resistance of *B. cinerea* to aniline pyrimidine fungicide pyrimidine is widespread, mainly high-resistant and medium-resistant strains. The resistance frequencies of *B. cinerea* to pyrimidine are different in different grape cultivation climate areas. The resistance frequencies of *B. cinerea* to pyrimidine are the highest in medium-temperature areas, reaching 62.5%. Resistance of Grape *B. cinerea* to pyrimidine amide is common, and the medium-sized strain is the main one. The highest frequency of resistance to pyridimide was 53.42%. Mutations of pyrimidine-resistant strains only occurred at the 272nd position of sdhB gene, and only H272R and H272Y mutations were found. Resistance of *B. cinerea* to enomorpholine has not been widespread. Four of 104 samples showed resistance, indicating that *B. cinerea* has begun to produce resistance to enomorpholine. At present, the use of enomorphine can effectively inhibit *B. cinerea*. The results of resistance analysis of grape gray mold to three insecticides were provided in this study, which could provide an effective reference for rational regulation of insecticide dosage and selection of insecticide varieties.

Author Contributions

Supervision, Hongmei Zhang and Huimin Jiang; writing, review and editing, Hongmei Zhang and Huimin Jiang; investigation, Hongmei Zhang; validation, Hongmei Zhang and Huimin Jiang; Advisor, Huimin Jiang. All authors have read and agreed to the published version of the manuscript.

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Ethical consideration

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

Interest conflict

The authors declare no conflict of interest.

References

1. Abuqamar S, Moustafa K, Tran LP. Mechanisms and strategies of plant defense against *Botrytis cinerea*. Crit Rev Biotechnol. 2017; 37(2): 262-274.
2. Aktaruzzaman M, Lee YG, Afroz T, Kim BS. The occurrence of postharvest gray-mold rot of sweet persimmon caused by *Botrytis cinerea* in Korea. Eur. J. Plant Pathol. 2017; 150(4): 1-7.
3. Ayoub F, Oujji NB, Chebli B, Ayoub M, Hafidi A, Salghi R. Antifungal effectiveness of fungicide and peroxyacetic acid mixture on the growth of *Botrytis cinerea*. Microb Pathog. 2017; 105(3): 74-80.
4. Chen Y, Liu T, Chen XR, Liu RJ. Simulation and evaluation method for pesticide residue pollution based on visual analysis techniques. Comput Simul. 2017; 34(10): 347-351.
5. Guo YG, Li CQ, Cui NX. Analysis and control of constant output current based on lcl-s type icpt system. J Pow Supply. 2017; 15(2): 109-114.
6. Hu Z, Shao S, Zheng C et al. Induction of systemic resistance in tomato against *Botrytis cinerea* by N-decanoyl-homoserine lactone via jasmonic acid signaling. Planta. 2018; 247 (5): 1217-1227.
7. Lecompte F, Nicot PC, Julie R, Abro MA, Raimbault AK, Félicie LL. Reduced susceptibility of tomato stem to the necrotrophic fungus *Botrytis cinerea* is associated with a specific adjustment of fructose content in the host sugar pool. Ann Bot. 2017; 119(5): 931-943.

8. Li Y, Shao X, Xu J. Effects and possible mechanism of tea tree oil against *Botrytis cinerea* and Penicilliu mexpansum in vitro and in vivo test. Can J Microbiol. 2017; 63(3): 219-227.
9. Liu C, Yan X, Wang M, Qin P, Cui ZN. Design, synthesis and fungicidal activity of novel 2-substituted aminocy cloalkylsulfonamides. Bioorg Medicinal Chem Letter. 2017; 27(2): 271-276.
10. Liu S, Hai F, Jiang J. Sensitivity to fludioxonil of *Botrytis cinerea* isolates from tomato in henan province of China and characterizations of fludioxonil-resistant mutants. J Phytopathol. 2017; 165(2): 98-104.
11. Lu XH, Jiao XL, Hao JJ, Chen AJ, Gao WW. Characterization of resistance to multiple fungicides in Botrytis cinerea populations from Asian ginseng in northeastern China. Eur J Plant Pathol 2016;144(3):467-76.
12. Minz-Dub A, Sharon A. The *Botrytis cinerea* pak kinase bccla4 mediates morphogenesis, growth and cell cycle regulating processes downstream of bcrac. Mol Microbiol. 2017; 104(3): 487-498.
13. Ren X, Song D, Zhuo L, Kong QJ. Effect of mint oil against *Botrytis cinerea* on table grapes and its possible mechanism of action. Eur J Plant Pathology. 2018; 151(2): 321-328.
14. Ren XY, Song DD, Zhuo L, Kong QJ. Correction to: effect of mint oil against *Botrytis cinerea* on table grapes and its possible mechanism of action. Eur J Plant Pathology. 2019; 153(3): 983-983.
15. Sharma E, Tayal P, Anand G. Functional analysis of diacylglycerol O -acyl transferase 2 gene to decipher its role in virulence of *Botrytis cinerea*. Curr Genetics. 2017; 64(4): 1-15.
16. Wang ZW, Jin P, Pu XY, Zhao G. Construction of power supply service risk identification model based on clustering analysis. Autom. Instrum. 2019; 232(2): 179-182.
17. Yu H, Du X. Differential regulation of calmodulin, phenylalanine ammonia-lyase, and salicylic acid in response to *Botrytis cinerea* infection in tomato with different Ca2+ concentrations. J Plant Nutr. 2018; 41(9): 1104-1118.
18. Zhang RF, Ye JM. Adaptive quasi-newton algorithm for blind source separation based on canonical correlation analysis. J Jilin Uni (Sci Edi). 2017; 55(3): 559-563.
19. Fallah F, Kahrizi D. Effect of Light Spectrum and Intensity on Growth of Grape (Vitis vinifera) Under In Vitro Conditions. J App Biotechnol Rep 2016; 3(4): 6; 495-499.
20. Dehghanian F, Kay M, Kahrizi D. A novel recombinant AzrC protein proposed by molecular docking and in silico analyses to improve azo dye's binding affinity. Gene. 2015; 569(2): 233–238.
21. Ghorbani T, Kahrizi D, Saeidi M, Arji I. Effect of sucrose concentrations on Stevia rebaudiana Bertoni tissue culture and gene expression. Cell Mol Biol 2017;63(8):33-7.
22. Masoumi SM, Kahrizi D, Rostami-Ahmadvandi H, Soorni J, Kiani S, Mostafaie A, Yari K. Genetic diversity study of some medicinal plant accessions belong to Apiaceae family based on seed storage proteins patterns. Mol Biol Rep 2012 Dec;39(12):10361-5.
23. Eruygur N, Ucar E, Akpulat HA, Shahsavari K, Safavi SM, Kahrizi D. In vitro antioxidant assessment, screening of enzyme inhibitory activities of methanol and water extracts and gene expression in Hypericum lydium. Mol Biol Rep 2019 Apr;46(2):2121-9.