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弥勒市葡萄灰霉病菌对4种杀菌剂的抗药性检测

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摘　要：【目的】灰霉病是葡萄生产过程中的一种重要病害，严重影响葡萄的产量和商品价值。明确弥勒市不同葡萄产区灰霉病菌对腐霉利、嘧霉胺、咯菌腈和氟吡菌酰胺的抗药状况，为葡萄灰霉病的化学防治提供理论依据。**【方法】**从弥勒市市辖的12个乡、镇（农场）采集葡萄灰霉病样本，分离和纯化得到197株灰霉病菌菌株。采用菌丝生长速率法测定197株灰霉病菌对4种农药的敏感性。**【结果】**葡萄灰霉病菌对腐霉利、嘧霉胺抗药频率分别为71.1%、100.0%，高抗频率分别为13.7%和23.8%；除在弥勒市弥阳地区检测到2株低抗咯菌腈菌株，其他地区未检测到抗咯菌腈菌株；分离的葡萄灰霉病菌均对氟吡菌酰胺敏感。**【结论】**弥勒地区葡萄灰霉病菌对腐霉利和嘧霉胺产生了严重的抗药性，腐霉利和嘧霉胺双重抗性频率为71.1%。在实际生产中，建议以咯菌腈或氟吡菌酰胺与生物农药交替使用进行葡萄灰霉病的防治。

关键词：葡萄；灰葡萄孢菌；腐霉利；嘧霉胺；咯菌腈；氟吡菌酰胺

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Evaluation on resistance of *Botrytis cinerea* to four fungicides in Mile county

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Abstract:【Objective】Grape grey mould, caused by *Botrytis cinerea*, brings about damage to grapes (*Vitis vinifera*) in vineyards throughout the world. The first signs of disease on grapes often appear during the bloom period, when the fungus attacks the flower. The infected berries may become covered with greyish-tan conidia of the fungus. Berry stalks and cluster stems may be invaded, causing them to shrivel, and berries that have split or have been punctured are often easily attacked by other organisms. Gray mold is one of the most serious fungal diseases in grape. Due to the lack of registered broad-spectrum compounds, effective control of grape grey mould is usually based on repeated fungicide applications during the growing season, especially in mild and humid climates. But the fungus is known to rapidly adapt to its environment and to develop fungicide resistance in the field. Because of these problems, the pesticide resistance management strategies have now been widely adopted, which restrict the usage of the same fungicide. However, increasing occurrence of isolates with low-level cross-resistance to various fungicides has been observed in Chinese vineyards. In Mile grape production, the following fungicide groups are in current use against *B. cinerea*: procymidone, pyrimethanil, fludioxonil and fluopyram. However, little is known about the pesticide resistance in *B. cinerea* populations from the grape in Mile county, which is the primary region for grape production in Yunnan province. The objectives of

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this study were to: (1) assess the resistance to procymidone and pyrimethanil and establish the resistance distribution of *B. cinerea* to procymidone and pyrimethanil in Mile county; (2) assess the stability of resistance to fludioxonil; (3) estimate the stability of resistance to fluopyram; (4) evaluate the efficacy of the four fungicides for controlling grape gray mold. 【Methods】The diseased samples were collected from Mile county in Yunan province. One hundred and ninety-seven strains of *B. cinerea* were isolated and purified. All isolates were then single-spore cultured and stored as mycelium plugs either in 15% glycerol at −80 ℃ for long-term storage or in sterile water at 4 ℃ for short-term storage. All isolates were screened for resistance to fungicides. Technical-grade fungicide was dissolved in dimethyl sulfoxide (DMSO) to make a 10 $mg \cdot mL^{-1}$ stock solution. To detect fungicide resistance, conidia from sporulating colonies growing on PDA were harvested in 5 mL of sterile ddH_2O. Final conidial concentration was adjusted at 2×10^5 $conidia \cdot mL^{-1}$ using a hemocytometer. For determining pathogen sensitivity to the pyrimethanil, a minimal medium instead of PDA was used containing 10 g glucose, 1.5 g K_2HPO_4, 2 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 2 g yeast extract, and 12.5 g agar per liter. Colony diameters were measured after incubation for 4 days at 20 ℃ in the dark and the inhibition of mycelial growth was calculated. The sensitivity assays were performed at the discriminatory concentrations of fungicides in the medium. Mycelial growth at a given discriminatory concentrations implies a fungicide-resistant phenotype. Resistance standards employed for procymidone, pyrimethanil, fludioxonil and fluopyram were 0.31, 0.091 1, 0.013 5 and 0.13 $mg \cdot L^{-1}$, respectively. Three replicate plates were used for each treatment and the experiments were performed three times for all tested fungicides. 【Results】The study showed the existence of procymidone- and pyrimethanil-resistant strains was at frequencies of 71.1% and 100.0%, respectively. High resistance frequencies of 13.7% and 23.8% were observed for procymidone and pyrimethanil, respectively. In procymidone-resistant strains, forty-four were low-resistant to procymidone (frequency: 23.4%), sixty-seven were medium-resistant to procymidone (frequency: 34.1%), and twenty-seven were high-resistant to procymidone (frequency: 13.7%). In pyrimethanil-resistant strains, thirty-six were low-resistant to pyrimethanil (frequency: 18.3%), one hundred and fourteen were medium-resistant to pyrimethanil (frequency: 57.9%), forty-seven were high-resistant to pyrimethanil (frequency: 23.8%). Frequencies of 71.1% were resistant to both procymidone and pyrimethanil. In the forty-seven pyrimethanil-high-resistance strains, thirty-eight were resistant to procymidone. Of thirty-eight strains, two were high-resistant to procymidone, sixteen were medium-resistant to procymidone, and twenty were low-resistant to procymidone. In this study, two isolates were found to be resistant to fludioxonil (frequency: 1.0%), while no strain resistant to fluopyram was detected. 【Conclusion】Our research has raised a serious concern in grape production in Mile county regarding the resistance of *B. cinerea* to the four fungicides most widely used to control gray mold. The present monitoring revealed the fungicide resistance phenotypes in *B. cinerea*, showing different levels of resistance to single fungicides and/or multiple resistance to different combinations of fungicides. The high levels of resistance observed with procymidone- and pyrimethanil-resistant strains and the high number of isolates with double-resistance to the two fungicides are alarming data. It's worth noting that the strains isolated were high-sensitive to fludioxonil and fluopyram, though two strains were resistant to fludioxonil. Thus, we suggest that procymidone and pyrimethanil should be restricted to use for controlling grape gray mold. We recommend that fludioxonil and fluopyram may be adopted for control and fungicide-resistance-management in *B. cinerea*.

Key words: Grape; *Botrytis cinerea*; Procymidone; Pyrimethanil; Fludioxonil; Fluopyram

葡萄灰霉病是由灰葡萄孢菌（*Botrytis cinerea*）引起葡萄的一种重要真菌病害[1]。灰葡萄孢菌寄主广泛，不同的专化型可以侵染多种作物，如葡萄、草莓、番茄和黄瓜。近年来，灰霉病在葡萄上的危害日渐加重。灰霉病菌主要侵染葡萄果实和叶片，直接影响葡萄的产量和品质。因缺少优质高产且抗病的品种，葡萄灰霉病仍是以化学农药防治为主。灰霉病菌繁殖速度快、产孢量大、易变异，致使其对多种农药产生了不同程度的抗药性，甚至多重抗药性。福州地区蔬菜作物灰霉病菌对腐霉利和嘧霉胺产生了较强的抗性，其中灰霉病菌对腐霉利高抗频率达48%，对嘧霉胺高抗频率达54%[2]。江苏丘陵地区田间草莓灰霉病菌种群对嘧菌酯（Azoxystrobin）和吡唑醚菌酯（Pyraclostrobin）产生了高水平抗药性[3]。辽宁省番茄灰霉病菌对腐霉利表现低、中抗药性和对嘧霉胺表现中、高抗药性[4]。在德国、法国等国家，来自葡萄的灰霉病菌对多菌灵（Carbendazim）、异菌脲（Iprodione）、环酰菌胺（Fenhexamid）产生了不同程度的抗药性[5-6]。我国一些地区的葡萄灰霉病菌已对多菌灵、腐霉利（Procymidone）、乙霉威（Diethofencarb）和嘧霉胺（Pyrimethanil）产生了抗药性[7-9]，甚至出现了“双抗”甚至“多抗”灰霉菌菌株[10]。

弥勒市葡萄种植始于20世纪50年代末，是我国最早种植葡萄的地区之一。2012年，弥勒葡萄获得国家地理标志证明商标。2020年，弥勒市葡萄种植面积为6.87 万hm^2，总产量16万t，产值7.4亿元。目前，葡萄产业已经成为当地农民增收致富的支柱产业。几十年来，弥勒市葡萄栽培方式随着农业现代化的不断发展，由最初的露地栽培，到避雨栽培，再到日光温室栽培为主。然而，在日光温室的栽培方式下，灰霉病危害日渐加重。目前，弥勒市葡萄灰霉病防治的常用农药以腐霉利、嘧霉胺、咯菌腈（Fludioxonil）和氟吡菌酰胺（Fluopyram）为主，但葡萄灰霉病菌是否已对这些药剂产生抗药性尚不清楚。为了明确弥勒地区葡萄灰霉病菌对这4种杀菌剂的抗药性状况，笔者从弥勒市葡萄主要产区采集、分离葡萄灰霉病菌，了解灰霉病菌的抗药性水平、地理分布。研究结果为指导葡萄灰霉病的药剂防治提供理论依据。

1　材料和方法

1.1　灰霉病菌分离

2020年3—5月分别在弥勒市市辖12个乡、镇（农场）的葡萄园采集葡萄灰霉病病叶。实验室内进行病原菌分离和单孢纯化后，共得到197株菌株，通过滤纸片法[11]保存于−20 ℃冰箱备用。

1.2　供试药剂和培养基

98%嘧霉胺原药、94%腐霉利原药、96%咯菌腈原药由沈阳化工研究院司乃国研究员馈赠。氟吡菌酰胺分析纯购于西格玛奥德里奇（上海）贸易有限公司。原药溶于适量二甲基亚砜（DMSO）中，置于4 ℃冰箱中保存。PDA培养基用于葡萄灰霉病对腐霉利、咯菌腈和氟吡菌酰胺敏感性测定；L-asp培养基[12]用于葡萄灰霉病菌对嘧霉胺敏感性的测定。

1.3　试验方法

采用菌丝生长速率法[9]测定葡萄灰霉病菌对嘧霉胺（区分剂量设置为0.1、1、10、50、100 $mg\cdot L^{-1}$）、腐霉利（区分剂量设置为0.1、5、50、100、500 $mg\cdot L^{-1}$）、咯菌腈（区分剂量设置为0.1、1、5、10、50 $mg\cdot L^{-1}$）和氟吡菌酰胺（区分剂量设置为0.1、5、50、100、200 $mg\cdot L^{-1}$）的敏感性。将葡萄灰霉病菌菌丝团（2 mm）接种于平板中央，以不含药剂平板为对照，每个处理3次重复。25 ℃恒温培养3 d，用十字交叉法测量各菌落直径。利用DPS软件[13]进行数据分析及计算药剂对病原菌的EC_{50}值。

1.4　葡萄灰霉病菌抗药性水平划分

以腐霉利敏感性基线0.31 $mg\cdot L^{-1}$[14]、嘧霉胺敏感性基线0.091 1 $mg\cdot L^{-1}$[12]、咯菌腈敏感性基线0.013 5 $mg\cdot L^{-1}$[15]和氟吡菌酰胺敏感性基线0.13 $mg\cdot L^{-1}$[16]进行判断。抗性水平=菌株EC_{50}÷敏感性基线。敏感菌株：抗性水平≤10；低抗菌株：10＜抗性水平≤50；中抗菌株：50＜抗性水平≤100；高抗菌株：抗性水平＞100。抗性频率/%=（抗性菌株数/总菌株数）×100。

2　结果与分析

2.1　葡萄灰霉病菌对腐霉利的抗药性水平

对197株灰霉病菌采用菌丝生长速率法进行了腐霉利抗药性测定（表1）。结果表明，弥勒地区葡萄灰霉病菌对腐霉利的抗药性菌株为140株，抗性频率为71.1%。其中低抗菌株44株，低抗频率为23.4%；中抗菌株67株，中抗频率为34.1%；高抗菌株27株，高抗频率13.7%。各地区葡萄灰霉病菌对腐霉利的抗性频率不同，其中弥勒朋普地区的抗性

表1 葡萄灰霉菌抗腐霉利类型及分布

Table 1 Resistance types of *Botrytis cinerea* strains isolated from grape against procymidone

采集地点 Location	菌株数 No. of strains	敏感菌株数 No. of sensitive strains	低抗菌株数 No. of low resistence strains	中抗菌株数 No. of medium resistence strains	高抗菌株数 No. of high resistence strains	抗性频率 Resistance frequency/%
弥阳 Miyang	49	15	11	17	6	69.4
新哨 Xinshao	53	19	12	18	4	64.2
竹园 Zhuyuan	22	7	6	8	1	68.2
朋普 Pengpu	11	6	3	2	0	45.5
巡检司 Xunjiansi	7	1	4	2	0	85.7
虹溪 Hongxi	5	0	2	2	1	100.0
五山 Wushan	3	0	2	1	0	100.0
江边 Jiangbian	2	0	0	2	0	100.0
东山 Dongshan	2	0	0	1	1	100.0
西一 Xiyi	3	0	1	1	1	100.0
西二 Xier	4	0	1	2	1	100.0
东风 Dongfeng	36	9	4	11	12	75.0
合计 Total	197	57	46	67	27	71.1

频率最低，为45.5%。

2.2 葡萄灰霉病菌对嘧霉胺的抗药性水平

所采集的菌株均对嘧霉胺产生了抗药性（图1）。其中，低抗菌株36株，低抗频率为18.3%；中抗菌株114株，中抗频率为57.9%；高抗菌株47株，高抗频率为23.8%。不同地区分离的灰霉病菌对嘧霉胺的抗药性水平不同。从五山、江边、东山和西一乡（镇）分离的葡萄灰霉病菌未检测到高抗菌株，其余地区均已出现高抗菌株。

2.3 葡萄灰霉病对咯菌腈和氟吡菌酰胺抗药性水平

将197株葡萄灰霉病菌进行咯菌腈和氟吡菌酰胺抗药性水平划分，发现195株对咯菌腈表现为敏感，仅在弥勒市弥阳地区检测到2个低抗菌株（抗性频率1.0%）；分离的葡萄灰霉病菌对氟吡菌酰胺均无抗性。

2.4 葡萄灰霉病对腐霉利和嘧霉胺的双重抗药性水平

因所分离的菌株均对嘧霉胺产生了抗药性，而灰霉病菌对腐霉利的抗性频率为71.1%。可以得出，田间已经出现腐霉利和嘧霉胺的双重抗药性菌株。为进一步分析田间是否已出现双高抗菌株，笔者对高抗嘧霉胺菌株和抗腐霉利菌株进行了交集分析（图2）。结果表明，既高抗嘧霉胺又抗腐霉利菌株为38株，在197株菌株中占比是19.3%；既高抗嘧霉胺又高抗腐霉利菌株为2株，占比是1.0%。

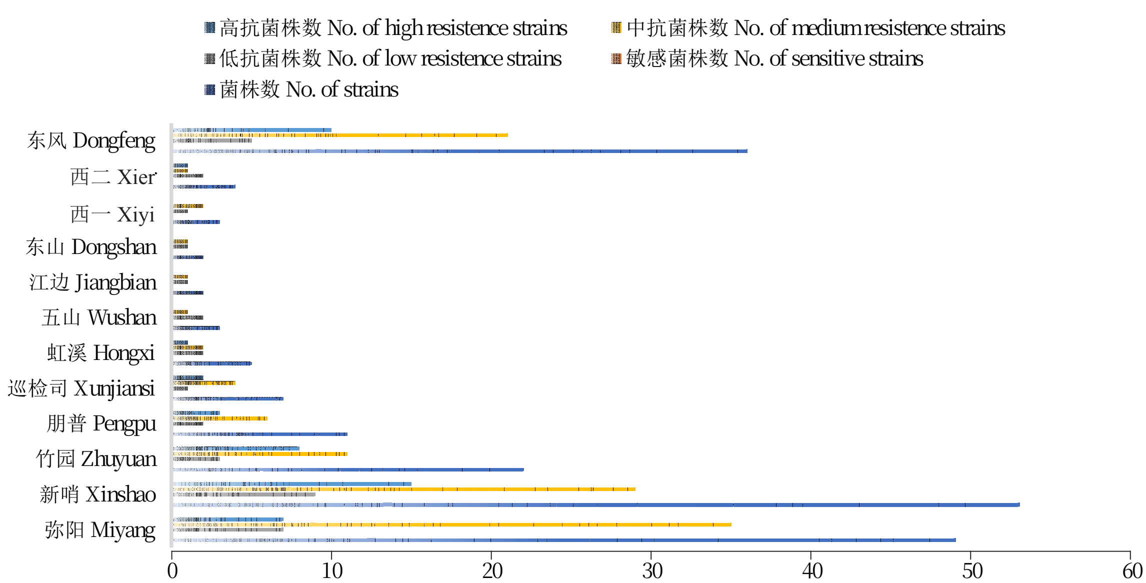
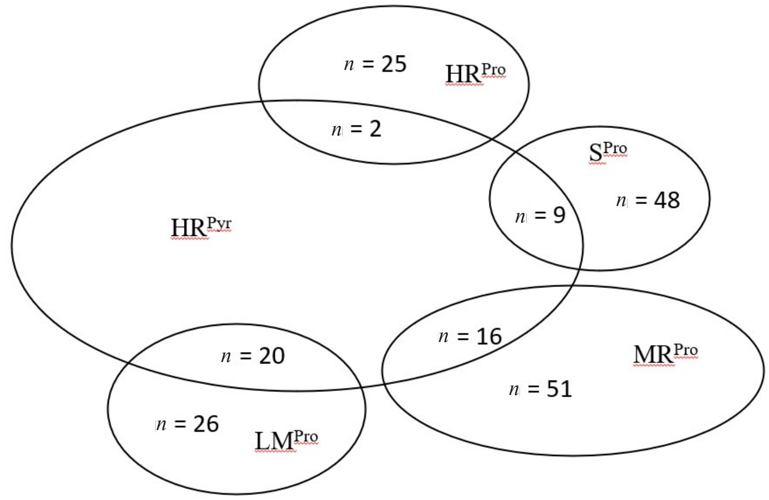


图1 葡萄灰霉病菌对嘧霉胺的抗药性检测

Fig. 1 Evaluation on resistance of grape gray mold pathogen *Botrytis cinerea* to pyrimethanil in Mile county



HRpyr. 高抗嘧霉胺菌株；Spro. 敏感腐霉利菌株；LRpro. 低抗腐霉利菌株；MRpro. 中抗腐霉利菌株；HRpro. 高抗腐霉利菌株；*n*. 菌株数。

HRpyr. Pyrimethanil-high-resistence strains；Spro. Procymidone-sensitive strains；LRpro. Procymidone-low-resistence strains；MRpro. Procymidone-medium-resistence strains；HRpro. Procymidone-high-resistence strains；*n*. Number of strains.

图 2　葡萄灰霉病菌对嘧霉胺和腐霉利的双重高抗药性分析

Fig. 2　Number of strains showing the phenotypes that are high-resistance to procymidone and resistant to pyrimethanil in Mile county

3　讨　论

弥勒葡萄种植的地区主要是弥阳、新哨、竹园、朋普、东风5个乡镇（农场），种植面积占弥勒葡萄种植总面积的90%以上。因此，笔者对这5个地区加大了样品采集数量，确保本研究结果能代表弥勒市葡萄灰霉病抗药性状况。试验结果表明，弥勒地区葡萄灰霉病菌对常用杀菌剂腐霉利和嘧霉胺已存在双重抗药性。嘧霉胺属于苯胺基嘧啶类杀菌剂。该类杀菌剂曾是防治灰霉病的一类高效杀菌剂，包括嘧菌环胺、嘧霉胺和嘧菌胺。然而，由于苯胺基嘧啶类杀菌剂作用靶点单一（抑制甲硫氨酸的合成），易使病菌产生抗性[17]。目前，灰霉病菌已经普遍对嘧霉胺产生了抗药性[4,9]。在农药压力选择下，中、高抗菌株将获得有利的选择优势，导致中、高抗嘧霉胺菌群逐渐成为主导种群。本研究结果得出弥勒葡萄灰霉病菌对腐霉利和嘧霉胺的抗性频率分别为71.1%和100.0%，这表明对腐霉利和嘧霉胺双重抗药性的菌株所占比例达71.1%。因此，在弥勒葡萄生产过程中建议停止使用嘧霉胺和腐霉利。

咯菌腈属于吡咯类杀菌剂，是防治葡萄灰霉病的常用药剂[18]。在弥勒市弥阳地区采集的2株灰霉病菌被检测到对咯菌腈产生了低抗药性。据报道，灰霉病菌对咯菌腈的抗性突变体的适合度低于野生菌株，不易形成优势种群[19-20]。因此，通过轮换用药或限定每年生长季的用药次数，咯菌腈在弥勒市葡萄灰霉病防治上应用前景依然广阔。氟吡菌酰胺是新型琥珀酸脱氢酶抑制剂（SDHI）类杀菌剂，具有内吸性和传导性，被广泛应用于灰霉病的防治[16]。笔者在本研究中未发现弥勒葡萄灰霉病对氟吡菌酰胺产生抗药性，因此，生产中葡萄灰霉病可采用咯菌腈或氟吡菌酰胺进行防治。

4　结　论

弥勒地区葡萄灰霉病菌对常用杀菌剂腐霉利和嘧霉胺已产生了严重的抗药性，且存在双重抗药性，建议暂停腐霉利和嘧霉胺在葡萄灰霉病防治中的使用。此外，葡萄灰霉病菌对咯菌腈已出现了低抗药性菌株，但抗性频率很低，建议生产中尽量避免此类药剂的单一使用，延缓葡萄灰霉病菌对此类杀菌剂抗药性的产生。葡萄灰霉病菌对氟吡菌酰胺抗性菌株尚未出现。因此，在生产实际中建议以咯菌腈、氟吡菌酰胺或与生物农药交替使用进行葡萄灰霉病防治。

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