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苹果炭疽叶枯病菌对3种杀菌剂的敏感性分析

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摘 要:【目的】了解我国苹果主产区苹果炭疽叶枯病菌(*Colletotrichum gloeosporioides*)对苯并咪唑类、甾醇脱甲基抑制剂类和咪唑类杀菌剂敏感性的现状,旨为苹果炭疽叶枯病的科学防治提供参考,以及为苹果炭疽叶枯病菌抗药分子机制研究提供理论依据。**【方法】**采用区分剂量法和菌丝生长速率法,对采自于我国苹果主产区的117个苹果炭疽叶枯病菌菌株进行甲基硫菌灵、戊唑醇、咪鲜胺的敏感性测定,并对随机抽测的28个菌株的β-微管蛋白基因(β-tubulin)进行序列分析。**【结果】**117个供试菌株对甲基硫菌灵的抗药性频率为100%,均为高水平抗性菌株(HR)。随机抽测24个菌株的β-tubulin基因,其中23个菌株的β-tubulin蛋白第198位氨基酸从谷氨酸(Glu)突变为丙氨酸(Ala),另外1个菌株ZG4-7的第200位氨基酸从苯丙氨酸(Phe)突变成酪氨酸(Tyr)。苹果炭疽叶枯病菌对戊唑醇的敏感性检测结果表明,戊唑醇对供试菌株的EC_{50}值(ρ,后同)为0~0.843 0 mg·L^{-1},平均EC_{50}值为0.155 5 mg·L^{-1},75.21%的菌株对戊唑醇表现出低水平抗药性,设置质量浓度为5 mg·L^{-1}时,对供试群体的平均抑制率为92.72%。苹果炭疽叶枯病菌供试群体对咪鲜胺的敏感性较强,平均EC_{50}值为0.011 9 mg·L^{-1},设置质量浓度为0.5 mg·L^{-1}时,咪鲜胺对供试群体的平均抑制率为95.02%。**【结论】**苹果炭疽叶枯病菌对苯并咪唑类杀菌剂甲基硫菌灵表现出高抗性;对DMIs类杀菌剂戊唑醇表现出低水平抗性,但已产生高水平抗性菌株;对咪唑类药剂咪鲜胺敏感性较强。

关键词: 苹果炭疽叶枯病菌;甲基硫菌灵;戊唑醇;咪鲜胺;β-tubulin基因;敏感性

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Detection of the sensitivity of *Colletotrichum gloeosporioides* to three fungicides

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Abstract:【Objective**】** *Colletotrichum gloeosporioides* (anamorph of *Glomerella cingulata*), a ubiquitous fungal pathogen, is the agent causing Glomerella leaf spot (GLS) on apples. Under favorable conditions, GLS can result in seventy-five percent defoliation by harvest, weakening trees and reducing yield. In recent years, an epidemic of GLSA broke out in most apple planting areas in China, because of an indefinite occurrence period and resulitn in a severe impairment. The disease is initiated when conidia attaches to the plants surfaces via wind or rain splash dispersal, where trhe conidia germinates and differentiates into a specialized infection structure called appressorium. After invasion, *C. gloeosporioides* grows biotrophically, proliferates into neighboring cells, turns into a necrotrophic development and eventually results in lesions on the plants. Planting a resistance cultivar is considered to be a key strategy and one of the most efficient methods of controlling plant diseases. Many quality cultivars derived from the "Delicious" group, including 'Gala' 'Gold Delicious', were widely planted. Some biocontrol products are applied to plants for protection and achieve good benefits of social and economic, but the commercial biocontrol product showed no effect on GLS in leaves and fruits. Therefore, this disease is primarily managed with chemical controls, such as benzimidazole fungicides, sterol demethylation in-

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hibitors (DMI) and imidazole. The severity of GLS can be reduced in the orchard by applying some fungicides beginning about 6 weeks after petal fall and continuing every 2 weeks until 2 to 3 weeks before harvest. Combinations of chelated zinc materials and dithiocarbamate fungicides improve control. However, while frequent applications are effective, they also increase production costs and can have damaging effects on the environment and human health. Moreover, plant pathogens can become resistant to fungicides and it can then fail as a control. For investigating the sensitivity of *C. gloeosporioides* to benzimidazole, DMI and imidazole, diseased leaves were collected from different apple gardens, and the obtained pathogen isolates were tested to see if they were resistance to benzimidazole, DMI and imidazole. This study could provide a theoretical basis for effective scientific controlling of GLSA.【Methods】One hundred and seventeen *C. gloeosporioides* strains were isolated from their diseased leaves, which were collected from different apple cultivars including 'Gala' 'Golden Delicious' 'Jonagold' 'Jinxiuhong' 'Jinhong' and 'K12' in different apple growing areas, such as Anyang, Henan; Zhengzhou, Henan; Zhaoyuan, Shandong; Qufu, Shandong; Yixian, Hebei; Xianyang, Shanxi and Dangshan, Anhui. The obtained isolates were separated using the single-spore method. The cultural characteristics of all the different isolates were studied on 6 cm diameter PLA plates. The morphology of the colony and conidia were studied after the plates were incubated for 10 days at 25 ℃ under a light microscope. For infection assays, a droplet (2 μL) of freshly harvested conidial suspensions was placed onto the obverse of the apple leaves ('Gold Delicious'), while 2 μL conidial suspensions were also inoculated into the wounded leaves by stabbing with a toothpick. Inoculated leaves were placed on plates containing water agar (12%). Disease lesions were examined at 4 days postinoculation. Each test was repeated three times with three repetitions for each time. In all, we obtained one hundred and seventeen *C. gloeosporioides* strains from five hundred and thirty-five isolates tested. To evaluate the resistance of *C. gloeosporioides* against thiophanate-methyl, prochloraz and tebuconazole, we tested the pathogen growth by using distinguishing dosages and the hyphae growth inhibition method. *C. gloeosporioides* strains were cultured on the PDA plates for seven days in the dark, then transferred into fresh PDA plates with different dosages, and the diameter of the colony was investigated on the seventh day. The effective inhibiting concentration (EC_{50}) and resistance frequency were calculated by using the DPS software. For investigating the mechanism of *C. gloeosporioides* resistance against thiophanate-methyl, β-tubulin gene sequences of 28 strains were amplified and sequenced. The fungal genomic DNA of the twenty-eight strains was isolated from the fungal vegetative hyphae using the cetyltrimethylammonium bromide (CTAB) procedure combined with RNase A and proteinase K treatment. PCR was conducted with *pfu* DNA polymerase, and PCR products were purified and sequenced by a bio-company. The partial amino acid sequences of β-tubulin proteins were aligned and analyzed by using the Bioedit software.【Results】The resistance frequency of the 117 isolates to thiophanate-methyl was 100%, which can be attributed to high level resistance. The β-tubulin gene sequence alignment of 27 of the selected 28 isolates indicated that GAA at codon 198 mutated into GCA, which resulted in a substitution of glutamic acid for alanine at codon 198. In addition, phenylalanine acid of the isolate Dj1-1-6 mutated into tyrosine acid at codon 200. The effective inhibiting concentrations (EC_{50}) of hyphen radial growth on tebuconazole ranged from 0 to 0.843 0 mg·L^{-1} with a mean value of 0.155 5 mg·L^{-1}. The sensitive assay showed that 75.21% of the 117 isolates had a low resistance to tebuconazole, and the hyphen inhibition rate was 92.72% at 5 mg·L^{-1}. All 117 isolates were highly sensitive to prochloraz with a mean value of 0.011 9 mg·L^{-1}, and the average inhibition rate of the colony growth was 95.02% at 0.5 mg·L^{-1}.【Conclution】This study shows that *C. gloeosporioides* has a high resistance to thiophanate-methyl, a low resistance to tebuconazole and a high sensitivity to prochloraz.

Key words: Glomerella leaf spot of apple; Thiophanate-methyl; Tebuconazole; Prochloraz; β-tubulin gene; Sensitivity

苹果炭疽叶枯病主要由胶孢炭疽菌(*Colletotrichum gloeosporioides*)引起,有性世代为子囊菌亚门围小丛壳菌(*Glomerel ciagulata*)[1]。苹果炭疽叶枯病菌主要侵染'金冠''嘎拉'和'乔纳金'等品种的叶片及果实,也危害'富士'和'秦冠'等品种的果实[2]。苹果炭疽叶枯病菌的发生会导致苹果树快速大量落叶,树势严重削弱,影响翌年产量[3]。1970年,苹果炭疽叶枯病在美国首次报道[4],目前在巴西、美国、中国等主要苹果生产国普遍流行发生,危害极其严重[5-8]。胶孢炭疽菌是一种普遍存在的植物病原菌,寄主非常广泛。其不同的专化型可以侵染1 000多种植物,包括水果、蔬菜、花卉和豆类[9]。因其与寄主的协同进化及其对环境的适应(如农药的选择压),进化或变异的菌株产生较快。因此,开展苹果炭疽叶枯病菌变异跟踪,特别是开展抗药性菌株监测研究具有重要的意义。

关于胶胞炭疽菌对多种农药产生抗药性的报道屡见不鲜。Kuma等[10]报道,印度主要杧果产区杧果炭疽菌(*C. gloeosporioides*)已对多菌灵和代森锰锌产生了严重的抗药性;杨叶等[11]报道海南省杧果炭疽菌(*C. gloeosporioides*)已对多菌灵产生了抗性;陈聃等[12]报道浙江省葡萄炭疽菌(*C. gloeosporioides*)已对甲基硫菌灵产生高水平抗药性,而对戊唑醇表现为低水平抗药性。以前的研究表明,植物病原真菌对甲基硫菌灵的抗性与β-微管蛋白的突变有关,特别是第198位氨基酸的突变(Glu突变为Leu)可使菌株获得高抗性,此外β-微管蛋白50或167或200位氨基酸的突变也会产生不同水平的抗性[13-14]。然而,关于苹果炭疽叶枯病菌是否已有抗药性菌株出现及其抗药性机制的研究未见报道。

目前,尚未登记苹果炭疽叶枯病菌杀菌剂产品。在生产中,用于苹果炭疽病防治的主要药剂有甲基硫菌灵、戊唑醇和咪鲜胺[15]。但由于苹果是我国第一大水果,种植非常广泛,各产区的用药习惯及环境条件存在一定的差异,为此,笔者从我国苹果的不同产区采集病叶,分离病原菌,并进一步进行不同产区、不同寄主来源苹果炭疽叶枯病菌对甲基硫菌灵、戊唑醇和咪鲜胺的敏感性差异分析。目的是检测我国苹果主产区的苹果炭疽叶枯病菌群体对多菌灵、戊唑醇和咪鲜胺的抗性水平,以期为苹果炭疽叶枯病菌的药剂选择与合理使用提供依据,为田间病原菌的抗药风险评估及监测提供信息。

1 材料和方法

1.1 材料

1.1.1 苹果炭疽叶枯病菌菌株 2016年7—9月,分别从安徽砀山、山东招远、山东曲阜、河南郑州、陕西咸阳、河南安阳、河北易县采集苹果炭疽叶枯病病叶68份。病原菌的分离及单孢纯化参照吴建圆等[2]的方法,略作修改。剪取叶片病健交界处2~3 mm组织块,在75%(φ)乙醇中浸泡30 s后,无菌水冲洗3~4次。用无菌滤纸吸干组织块,置于含100 mg·L^{-1}氯霉素的PDA平板上,25 ℃黑暗培养3 d,待长出菌丝后,从菌落边缘挑取菌丝,转移到新的PDA平板上,25 ℃黑暗培养5 d后,刮去菌落表面菌丝,继续培养至10 d,进行单孢分离。50 μL分生孢子悬浮液(每mL 1×10^{4}个)涂布于2%(ω)水琼脂平板上,于25 ℃下培养10 h后,在解剖显微镜下,挑取已萌发的单个分生孢子于PDA平板,共得到535个菌株。经菌落形态、分生孢子形态观察及致病性测验,共获得供试苹果炭疽叶枯病菌菌株117个(表1)。

1.1.2 供试药剂 90%(ω,后同)甲基硫菌灵(Thiophanate-methyl)原药、85%戊唑醇(Tebuconazole)原药和87%咪鲜胺(Prochloraz)原药由沈阳化工研究院司乃国研究员馈赠。所用原药用二甲基亚砜(DMSO)进行配制。

1.2 方法

1.2.1 苹果炭疽叶枯病菌对甲基硫菌灵、咪鲜胺和戊唑醇的抗药性定性测定 采用区分剂量法[16]。各供试菌株分别在不含有杀菌剂的PDA平板上,25 ℃黑暗培养7 d,挑取(灭菌牙签)生长势相同的菌丝(直径约0.1 cm的菌丝团),转移到含有不同剂量杀菌剂的PDA平板上(以不含有杀菌剂的PDA平板为对照,每个处理3次重复),25 ℃黑暗培养7 d后观测。

甲基硫菌灵的剂量(ρ,后同)分别为0、5、20、100 mg·L^{-1},敏感性划分标准参考杨叶等[11]的报道:不能在含5 mg·L^{-1}甲基硫菌灵的PDA平板上生长的为敏感菌株(S);能在5 mg·L^{-1}而不能在20 mg·L^{-1}的PDA平板上生长的为甲基硫菌灵低水平抗性菌株(LR);能在20 mg·L^{-1}而不能在100 mg·L^{-1}的PDA平板上生长的为中等水平抗性菌株(MR);能在100 mg·L^{-1}的PDA平板上生长的为高水平抗性菌株(HR)。

戊唑醇的剂量分别为0、5、20 mg·L^{-1},敏感性划

表1　供试苹果炭疽叶枯病菌菌株

Table 1 *Colletotrichum gloeosporioides* isolates used in this study

采集地点 Collection area	寄主品种 Host cultivar	菌株编号 Isolate code
安徽砀山 Dangshan, Anhui	嘎拉 Gala	AG1-11, AG12-5, AG14-1, AG14-4, AG19-1, AG21-4, AG2-4, AG24-1, AG2-5, AG27-13, AG29-1, G32-4, AG34-6, AG35-5, AG4-5, AG5-3, AG9-5
	金帅 Golden Delicious	AJ23-4, AJ30-1, AJ32-1, AJ34-3, AJ36-3, AJ44-5-C1, AJ44-5-C5, AJ44-5-C6, AJ45-5-7, AJ45-5-8, AJ49-3-1-1, AJ52-4-1, AJ58-5, AJ62-1, AJ65-5, AJ7-6
	乔纳金 Jonagold	AQ12-5-C3, AQ12-5-C6, AQ13-5-2, AQ14-3, AQ16-15-1, AQ16-7, AQ21-3, AQ31-4, AQ32-5, AQ32-9, AQ45-10, AQ50-4-1, AQ50-4-5, AQ50-5, AQ52-1-5, AQ5-5, AQ6-C9, AQ9-4
山东招远 Zhaoyuan, Shandong	嘎拉 Gala	DG10-1, DG12-1, DG14-2, DG18-1, DG18-C4, DG5-2, DG9-1, DG9-3
山东曲阜 Qufu, Shandong	金红 Jinhong	DJ10-C2, DJ1-1-6, DJ11-C2, DJ12-C1, DJ12-C2, DJ2-C2, DJ3-C1, DJ4-2-1, DJ5-C1, DJ6-2-1, DJ7-3, DJ7-5, DJ9-1-1, DJ9-1-2
陕西咸阳 Xianyang, Shaanxi	嘎拉 Gala	WG11-9, WG14-1, WG14-2, WG20-1, WG20-2, WG38-6-C2, WG38-6-C6, WG38-6-C7, WG41-5, WG46-2-C1, WG46-2-C5, WG9-5
河南安阳 Anyang, Henan	华美 Huamei	HM3-2, HM3-C1, HM4-1
	K12	KM1-1, KM3-1
	金秀红 Jinxiuhong	XH1-1, XH3-1, XH4-1, XH4-4, XH4-6
	嘎拉 Gala	XG1-1, XG2-2, XG3-2, XG6-1
河南郑州 Zhengzhou, Henan	嘎拉 Gala	ZG3-1, ZG3-3, ZG4-1, ZG4-7, ZG5-3, ZG6-5, ZG7-5, ZG8-5
河北易县 Yixian, Hebei	乔纳金 Jonagold	HQ11-7-C2, HQ11-7-C3, HQ11-7-C4, HQ4-1-C5, HQ5-1-C3, HQ6-1-C1, HQ6-1-C7, HQ8-1-C3, HQ8-1-C4, HQ8-1-C7

分标准：不能在含5 mg·L^{-1}戊唑醇的PDA平板上生长的为敏感性菌株（S）；能在5 mg·L^{-1}上生长而不能在20 mg·L^{-1}的PDA平板上生长的为低水平抗性菌株（LR）；能在20 mg·L^{-1}的PDA平板上生长的为高水平抗性菌株（HR）。

咪鲜胺的剂量分别为0、1、5 mg·L^{-1}，敏感性划分标准：不能在含1 mg·L^{-1}咪鲜胺的PDA平板上生长的为敏感性菌株（S）；能在1 mg·L^{-1}上生长而不能在5 mg·L^{-1}的PDA平板上生长的为低水平抗性菌株（LR）；能在5 mg·L^{-1}的PDA平板上生长的为高水平抗性菌株（HR）。

1.2.2　苹果炭疽叶枯病菌对咪鲜胺和戊唑醇的敏感性测定　（1）有效抑制中质量浓度（EC_{50}）测定。采用菌丝生长速率法[17]。戊唑醇的质量浓度分别为0、1、5、10、20、40 mg·L^{-1}；咪鲜胺的质量浓度分别为0、0.1、0.5、1 mg·L^{-1}，参照1.2.1的方法进行。25 ℃黑暗培养7 d后，测量每个处理的菌落直径（cm），取平均值。计算杀菌剂对苹果炭疽叶枯病菌的抑制率（%），通过质量浓度对数值（X）和抑制率概率值（Y）之间的线性回归关系和毒力回归方程Y=aX+b，求有效抑制中质量浓度EC_{50}值。

抑制率/%=[(对照的菌落直径−处理的菌落直径)/(对照的菌落直径−0.1)]×100

（2）敏感性分布图制作。苹果炭疽叶枯病菌株对测试药剂的敏感性EC_{50}值从低到高分成8个左右的区间，统计EC_{50}值在各个区间的菌株占整个菌株群体的百分比（%），即频率。以EC_{50}值为X轴，相应的频率（%）为Y轴，即得苹果炭疽叶枯病菌株对杀菌剂的敏感性分布图。

1.3　数据处理

用DPS软件进行差异显著性分析[18]。由3次重复测验计算出每组数据的平均值和标准差，在α=0.05和α=0.01水平上，进行差异显著性分析，采用Duncan氏新复极差法（DMRT）进行标注。

1.4　苹果炭疽叶枯病菌β-tubulin基因序列的扩增和测序

随机选取24株甲基硫菌灵高抗菌株，提取基因组DNA。DNA提取参照真菌基因组DNA快速抽提取试剂盒（上海生工生物）说明书。各苹果炭疽叶枯病菌菌株分别在不含杀菌剂的PDA平板上，25 ℃黑暗培养7 d，无菌钥匙刮取菌丝，移入无菌的1.5 mL离心管中，液氮速冻，研磨成粉末状，具体过程参照说明书进行。

扩增引物的设计，参照已经报道的维管蛋白基因通用引物[19]，P3-1（5-CCTATCCTCGGTCAAGCCCA-3）和P3-2（5-GAAGCCCATGTTCTG-

GCAAA-3)，结合笔者实验室苹果炭疽叶枯病菌全基因组序列，利用NDAMAN设计引物V1和V2。引物委托生工生物工程股份有限公司(上海)合成。微管蛋白基因的PCR扩增体系为50 μL，其中引物(10 μmol · L^{-1})各2 μL，模板DNA 2 μL，5×Trans Start® FastPfu Buffer 10 μL，2.5 mmol · L^{-1} dNTPs 5 μL，Trans Start® FastPfu DNA Polymerase 1 μL，ddH_2O 28 μL。扩增程序：95 ℃预变性2 min，95 ℃变性20 s，55 ℃退火20 s，72 ℃延伸90 s，35个循环，72 ℃延伸5 min。PCR产物7 μL，经1.0%(ω)琼脂糖凝胶电泳检测，采用TaKaRa公司的DNA回收试剂盒对各菌株的PCR目的片段进行回收和纯化。目的片段末端连接三磷酸腺嘌呤脱氧核苷酸dATP，并克隆于pUCm-T载体中。分别转化*Escherichia coli* T1 (Trans1-T1 Phage Resistant)，涂布在含氨苄青霉素的LB平板上，过夜培养后挑选白色菌落进行小量培养，并提取质粒DNA，通过PCR扩增鉴定重组质粒。菌液委托生工生物工程股份有限公司(上海)测序，测序结果用Bioedit软件进行数据分析。

2 结果与分析

2.1 苹果炭疽叶枯病菌对甲基硫菌灵的敏感性分析

2.1.1 抗性频率 甲基硫菌灵质量浓度为100 mg·L^{-1}时，对苹果炭疽叶枯病菌菌丝的平均抑制率为41.64%；当质量浓度增加至800 mg·L^{-1}时，平均抑制率仅为45.95%(表2)。117个苹果炭疽叶枯病菌株均能在含有100 mg·L^{-1}甲基硫菌灵的PDA平板上生长(表3)，区分剂量法测定结果表明，苹果炭疽叶枯病菌已经对甲基硫菌灵产生高水平抗性，抗性频率达到100%。

表2 不同杀菌剂对苹果炭疽叶枯病菌的抑制率

Table 2 Inhibition rate of different fungicides to *Colletotrichum gloeosporioides*

杀菌剂 Fungicides	ρ/(mg·L^{-1})										
	0.1	0.5	1	5	10	20	40	100	200	400	800
咪鲜胺 Prochloraz	84.33	95.02	97.31	100.00	-	-	-	-	-	-	-
戊唑醇 Tebuconazole	-	-	74.02	92.60	93.73	98.40	99.44	-	-	-	-
甲基硫菌灵 Thiophanatemethyl	-	-	31.48	42.17	37.38	38.68	37.29	41.64	43.07	30.54	45.95

注："-"表示没有检测。

Note: "-" indicates no detection.

表3 供试苹果炭疽叶枯病菌株对三种杀菌剂的敏感性

Table 3 Sensitivity of *Colletotrichum gloeosporioides* against three fungicides

菌株编号 Isolate code	咪鲜胺 Prochloraz			戊唑醇 Tebuconazole			甲基硫菌灵 Thiophanate-methyl	
	有效抑制中质量浓度 Effective inhibiting concentration, EC_{50}/(mg·L^{-1})	致死质量浓度 Lethal concentration/(mg·L^{-1})	敏感性 Sensibility	有效抑制中质量浓度 Effective inhibiting concentration, EC_{50}/(mg·L^{-1})	致死质量浓度 Lethal concentration/(mg·L^{-1})	敏感性 Sensibility	致死质量浓度 Lethal concentration/(mg·L^{-1})	敏感性 Sensibility
AG1-11	-	1	S	0.193 6	20	LR	>800	HR
AG12-5*	-	1	S	-	1	S	>800	HR
AG14-1	0.001 0	2	S	0.155 7	20	LR	>800	HR
AG14-4*	-	2	S	0.200 0	10	LR	>800	HR
AG19-1	0.000 4	2	S	0.255 4	10	LR	>800	HR
AG21-4*	-	1	S	0.099 2	40	HR	>800	HR
AG2-4	-	1	S	0.515 5	10	LR	>800	HR
AG24-1	0.000 0	2	S	0.180 6	20	LR	>800	HR
AG2-5	-	1	S	0.407 0	10	LR	>800	HR
AG27-13*	-	1	S	0.008 6	>40	HR	>800	HR
AG29-1	0.000 8	2	S	0.088 3	40	HR	>800	HR
AG32-4	0.004 3	1	S	0.006 3	20	LR	>800	HR
AG34-6	0.001 5	2	S	0.024 3	20	LR	>800	HR
AG35-5	0.013 4	2	S	0.164 9	20	LR	>800	HR
AG4-5*	0.003 7	2	S	0.021 1	20	LR	>800	HR
AG5-3	0.000 0	2	S	0.058 6	40	HR	>800	HR

表3（续） Table 3（continued）

菌株编号 Isolate code	咪鲜胺 Prochloraz			戊唑醇 Tebuconazole			甲基硫菌灵 Thiophanate-methyl	
	有效抑制中质量浓度 Effective inhibiting concentration, EC_{50}/(mg·L^{-1})	致死质量浓度 Lethal concentration/(mg·L^{-1})	敏感性 Sensibility	有效抑制中质量浓度 Effective inhibiting concentration, EC_{50}/(mg·L^{-1})	致死质量浓度 Lethal concentration/(mg·L^{-1})	敏感性 Sensibility	致死质量浓度 Lethal concentration/(mg·L^{-1})	敏感性 Sensibility
AG9-5	0.000 1	2	S	-	5	S	>800	HR
AJ23-4	0.086 4	1	S	0.186 1	10	LR	>800	HR
AJ30-1	-	1	S	0.164 6	40	HR	>800	HR
AJ32-1	0.008 3	2	S	0.117 1	20	LR	>800	HR
AJ34-3	-	1	S	0.147 7	20	LR	>800	HR
AJ36-3	-	1	S	0.050 7	40	HR	>800	HR
AJ44-5-C1	-	1	S	0.060 0	40	HR	>800	HR
AJ44-5-C5	0.000 0	1	S	0.051 2	40	HR	>800	HR
AJ44-5-C6	-	1	S	0.259 4	10	LR	>800	HR
AJ45-5-7	-	1	S	0.015 7	10	LR	>800	HR
AJ45-5-8*	0.000 0	2	S	0.146 9	>40	HR	>800	HR
AJ49-3-1	-	1	S	0.036 4	10	LR	>800	HR
AJ52-4-1	0.000 1	1	S	0.015 4	20	LR	>800	HR
AJ58-5*	-	1	S	0.040 8	>40	HR	>800	HR
AJ62-1	-	1	S	0.001 3	20	LR	>800	HR
AJ65-5	0.011 6	2	S	0.000 0	20	LR	>800	HR
AJ7-6	0.020 2	2	S	0.148 8	10	LR	>800	HR
AQ12-5-C3	-	1	S	0.295 0	10	LR	>800	HR
AQ12-5-C6	0.032 8	2	S	0.194 7	20	LR	>800	HR
AQ13-5-2	-	1	S	0.098 5	>40	HR	>800	HR
AQ14-3	0.013 5	1	S	0.158 7	20	LR	>800	HR
AQ16-15-1	-	1	S	0.076 5	>40	HR	>800	HR
AQ16-7	-	2	S	0.120 3	20	LR	>800	HR
AQ21-3	-	1	S	0.049 9	40	HR	>800	HR
AQ31-4	-	1	S	0.193 4	20	LR	>800	HR
AQ32-5	-	1	S	0.092 1	40	HR	>800	HR
AQ32-9	0.073 9	2	LR	0.009 3	40	HR	>800	HR
AQ45-10	0.000 0	1	S	0.123 6	10	LR	>800	HR
AQ50-4-1	0.000 4	1	S	0.155 8	20	LR	>800	HR
AQ50-4-5*	0.007 9	1	S	0.061 2	>40	HR	>800	HR
AQ50-5	0.028 6	1	S	0.139 1	10	LR	>800	HR
AQ52-1-5	0.000 0	1	S	0.216 1	10	LR	>800	HR
AQ5-5	0.013 5	1	S	0.186 7	10	LR	>800	HR
AQ6-C9	-	1	S	0.320 3	10	LR	>800	HR
AQ9-4	0.000 2	2	S	0.381 0	20	LR	>800	HR
DG10-1*	0.000 3	1	S	0.302 7	20	LR	>800	HR
DG12-1	0.000 0	1	S	0.127 5	20	LR	>800	HR
DG14-2*	0.010 0	1	S	0.216 6	20	LR	>800	HR
DG18-1*	0.000 1	1	S	0.433 9	10	LR	>800	HR
DG18-C4	0.002 1	2	S	0.138 4	10	LR	>800	HR
DG5-2	0.004 6	2	S	0.146 9	10	LR	>800	HR
DG9-1	-	1	S	0.251 6	10	LR	>800	HR
DG9-3	-	1	S	0.530 1	10	LR	>800	HR
DJ10-C2	-	1	S	0.265 4	10	LR	>800	HR
DJ1-1-6*	-	1	S	0.218 6	10	LR	>800	HR
DJ11-C2	-	1	S	0.005 7	>40	HR	>800	HR
DJ12-C1	0.027 1	1	S	0.098 5	10	LR	>800	HR
DJ12-C2	0.000 4	1	S	0.180 6	20	LR	>800	HR
DJ2-C2	0.002 0	2	S	0.272 0	10	LR	>800	HR
DJ3-C1*	-	1	S	0.158 2	20	LR	>800	HR
DJ4-2-1*	-	1	S	0.206 3	20	LR	>800	HR
DJ5-C1	-	1	S	0.346 5	20	LR	>800	HR
DJ6-2-1	0.018 8	2	S	0.001 7	20	LR	>800	HR

表 3（续） Table 3（continued）

菌株编号 Isolate code	咪鲜胺 Prochloraz			戊唑醇 Tebuconazole			甲基硫菌灵 Thiophanate-methyl	
	有效抑制中质量浓度 Effective inhibiting concentration, EC_{50}/(mg·L^{-1})	致死质量浓度 Lethal concentration/(mg·L^{-1})	敏感性 Sensibility	有效抑制中质量浓度 Effective inhibiting concentration, EC_{50}/(mg·L^{-1})	致死质量浓度 Lethal concentration/(mg·L^{-1})	敏感性 Sensibility	致死质量浓度 Lethal concentration/(mg·L^{-1})	敏感性 Sensibility
DJ7-3	0.035 1	2	S	0.037 0	20	LR	>800	HR
DJ7-5	0.023 2	2	S	0.255 0	10	LR	>800	HR
DJ9-1-1	0.019 1	2	S	0.219 2	20	LR	>800	HR
DJ9-1-2*	0.039 5	2	S	0.374 9	10	LR	>800	HR
HM3-2*	-	2	S	0.036 6	20	LR	>800	HR
HM3-C1	0.000 3	2	S	0.033 1	20	LR	>800	HR
HM4-1	-	1	S	0.046 7	40	HR	>800	HR
HQ11-7-C2	-	1	S	0.546 2	10	LR	>800	HR
HQ11-7-C3	0.012 3	1	S	-	5	S	>800	HR
HQ11-7-C4	-	1	S	0.699 5	10	LR	>800	HR
HQ4-1-C5	0.001 7	1	S	0.399 1	10	LR	>800	HR
HQ5-1-C3	-	1	S	0.335 9	10	LR	>800	HR
HQ6-1-C1	-	1	S	0.105 3	10	LR	>800	HR
HQ6-1-C7	0.002 7	2	S	0.218 3	20	LR	>800	HR
HQ8-1-C3	-	1	S	0.843 0	10	LR	>800	HR
HQ8-1-C4	-	1	S	0.236 0	10	LR	>800	HR
HQ8-1-C7	0.009 3	1	S	0.189 0	20	LR	>800	HR
KM1-1*	-	1	S	0.373 9	10	LR	>800	HR
KM3-1*	0.007 9	1	S	0.326 7	10	LR	>800	HR
WG11-9	-	1	S	0.263 7	20	LR	>800	HR
WG14-1*	-	1	S	0.223 1	20	LR	>800	HR
WG14-2*	-	1	S	0.040 6	>40	HR	>800	HR
WG20-1	-	1	S	0.268 3	20	LR	>800	HR
WG20-2	-	1	S	0.254 0	20	LR	>800	HR
WG38-6-C2	-	1	S	0.158 5	20	LR	>800	HR
WG38-6-C6	-	1	S	0.701 8	10	LR	>800	HR
WG38-6-C7	-	1	S	-	5	S	>800	HR
WG41-5	-	1	S	0.051 6	10	LR	>800	HR
WG46-2-C1	-	1	S	0.163 9	20	LR	>800	HR
WG46-2-C5	-	1	S	0.133 1	20	LR	>800	HR
WG9-5	-	1	S	0.198 6	20	LR	>800	HR
XG1-1	0.028 0	1	S	0.137 8	20	LR	>800	HR
XG2-2*	0.014 3	1	S	-	5	S	>800	HR
XG3-2	0.017 5	1	S	0.011 6	>40	HR	>800	HR
XG6-1*	-	1	S	0.010 2	40	HR	>800	HR
XH1-1*	0.000 0	1	S	0.129 8	20	LR	>800	HR
XH3-1*	0.000 0	1	S	0.074 0	20	LR	>800	HR
XH4-1	0.007 0	2	S	0.019 0	40	HR	>800	HR
XH4-4	0.000 1	1	S	0.066 6	20	LR	>800	HR
XH4-6	0.000 0	2	S	0.242 7	10	LR	>800	HR
ZG3-1	-	1	S	0.050 1	40	HR	>800	HR
ZG3-3	0.076 9	1	S	0.154 2	20	LR	>800	HR
ZG4-1	0.032 7	1	S	0.504 0	20	LR	>800	HR
ZG4-7*	0.010 5	1	S	0.237 7	20	LR	>800	HR
ZG5-3	-	1	S	0.230 0	20	LR	>800	HR
ZG6-5	0.011 1	2	S	0.236 0	20	LR	>800	HR
ZG7-5	0.003 1	2	S	0.012 3	20	LR	>800	HR
ZG8-5*	0.000 0	2	S	0.128 8	20	LR	>800	HR

注：S. 敏感性菌株；LR. 低水平抗性菌株；HR. 高水平抗性菌株；*. 微管蛋白基因序列比对的菌株；−. 过于敏感菌株。

Note: S. Sensitivity strains; LR. Low resistance strains; HR. High resistance strains; *. The strains were used in the analysis of β-tubulin gene sequence; −. High sensitivity strains.

2.1.2　β-tubulin微管蛋白基因序列分析　通过引物V1和V2，扩增24个甲基硫菌灵高水平抗性苹果炭疽叶枯病菌株的微管蛋白基因片段，经测序后得到长度约2.0 kb的序列，与苹果炭疽叶枯病菌微管蛋白序列比对，利用Bioedit分析。其中23个菌株的β-tubulin基因第198位密码子从GAA突变为GCA，导致第198位氨基酸从谷氨酸（E）突变为丙氨酸（A），另外1个菌株ZG4-7的第200位密码子从TTC突变为TAC，导致第200位氨基酸从苯丙氨酸（F）突变成酪氨酸（Y）（图1）。

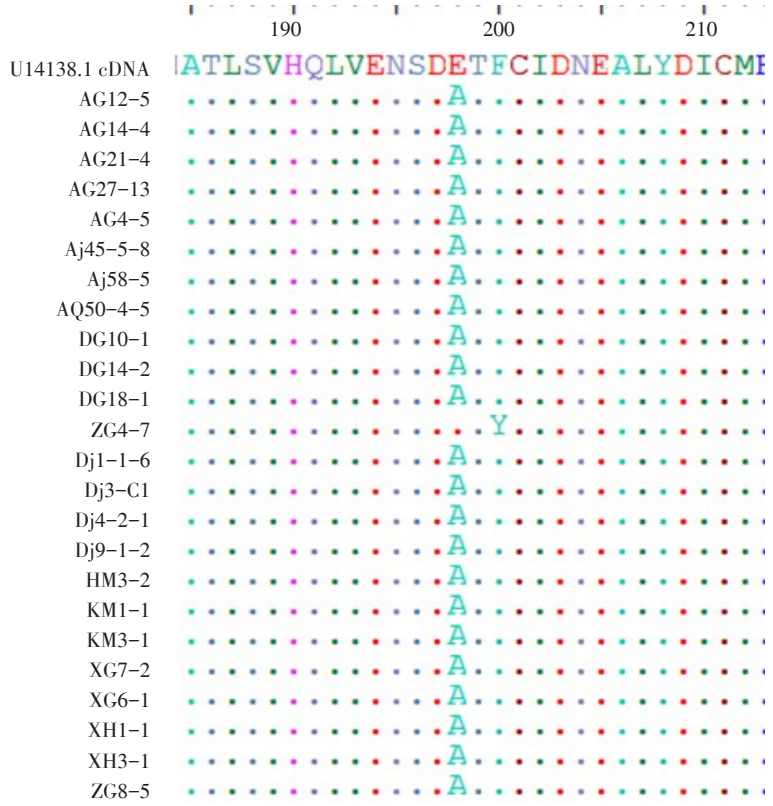


图1　β-tubulin蛋白部分氨基酸序列比对

Fig. 1　Alignment analysis of the partial amino acid sequences of β-tubulin protein

2.2　苹果炭疽叶枯病菌对咪鲜胺的敏感性分析

苹果炭疽叶枯病菌在含有0.1 mg·L^{-1}咪鲜胺的PDA平板上生长受到抑制，平均抑制率为84.33%，在含1 mg·L^{-1}咪鲜胺的PDA平板上生长受到明显抑制，平均抑制率达到97.31%。在含5 mg·L^{-1}咪鲜胺的PDA平板上不生长（表2）。利用菌丝生长速率法分析，咪鲜胺对苹果炭疽叶枯病菌株的EC_{50}均值为0.011 9 mg·L^{-1}，依据FAO标准，咪鲜胺EC_{50}值大于0.058 0 mg·L^{-1}的苹果炭疽叶枯病菌为咪鲜胺抗性菌株，苹果炭疽叶枯病菌对咪鲜胺的抗性频率为4.84%（图2）。依据区分剂量法和菌丝生长速率法，可将供试的苹果炭疽叶枯病菌对咪鲜胺的敏感性划分为2种类型[16]，敏感菌株不能在1 mg·L^{-1}咪鲜胺的PDA平板上生长，EC_{50}值小于0.058 0 mg·L^{-1}；低水平抗性菌株能在1 mg·L^{-1}咪鲜胺的PDA平板上生长，但不能在2 mg·L^{-1}咪鲜胺的PDA平板上生长，EC_{50}值大于0.058 0 mg·L^{-1}。结果表明，仅有AQ32-9表现为低抗（表3），没有发现高水平抗性菌株。

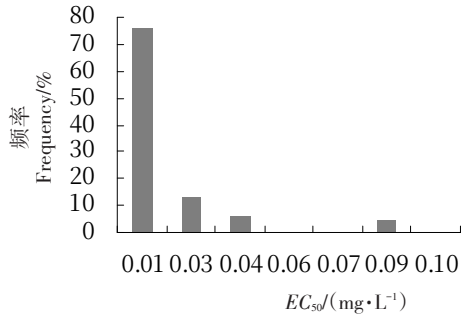
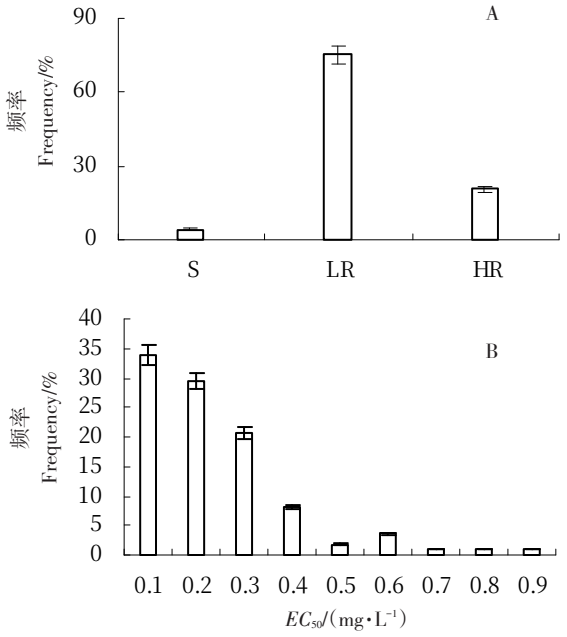


图2　苹果炭疽叶枯病菌对咪鲜胺的敏感性频率（EC_{50}）

Fig. 2　Sensitive frequency of *Colletotrichum gloeosporioides* against prochloraz (EC_{50})

2.3 苹果炭疽叶枯病菌对戊唑醇的敏感性分析

2.3.1 抗性频率 苹果炭疽叶枯病菌在含有1 mg·L^{-1}戊唑醇的PDA平板上生长受到抑制，平均抑制率为74.02%，在含有5 mg·L^{-1}戊唑醇的PDA平板上生长受到明显抑制，平均抑制率为92.6%（表2）。利用区分剂量法分析，75.2%的菌株对戊唑醇已产生低水平抗性，20.5%的菌株对戊唑醇已产生高水平抗性（图3-A）。利用菌丝生长速率法分析，戊唑醇对苹果炭疽叶枯病菌株的EC_{50}值为0~0.843 0 mg·L^{-1}，平均EC_{50}值为0.183 7 mg·L^{-1}，戊唑醇对苹果炭疽叶枯病菌株的EC_{50}值为0~0.2 mg·L^{-1}，频率为63.4%（图3-B）。不同菌株之间对戊唑醇的敏感性差异较大（表3）。



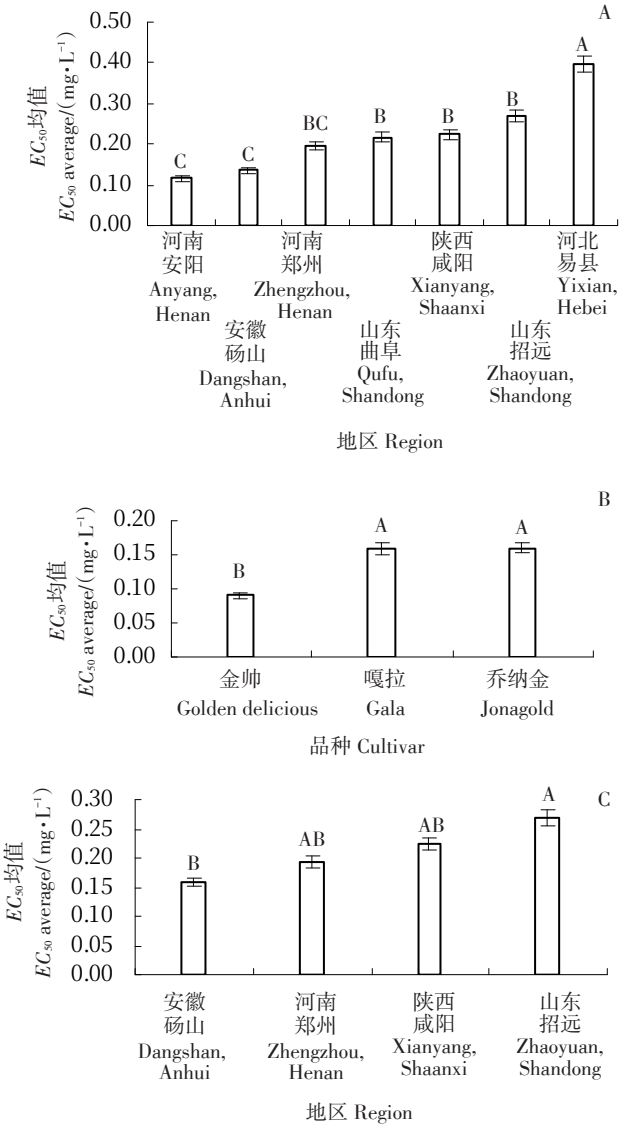
S. 敏感性菌株；LR. 低水平抗性菌株；HR. 高水平抗性菌株。

S. Sensitivity strains; LR. Low resistance strains; HR. High resistance strains.

图3 苹果炭疽叶枯病菌对戊唑醇的敏感性频率

Fig. 3 Sensitive analysis of *Colletotrichum gloeosporioides* against tebuconazole

2.3.2 苹果炭疽叶枯病菌对戊唑醇的敏感性差异分析 不同地区不同寄主品种分离得到的苹果炭疽叶枯病菌对戊唑醇的敏感性有显著差异（图4-A）。戊唑醇对河北易县苹果炭疽叶枯病菌株的平均EC_{50}值较高，为0.396 9 mg·L^{-1}；对河南安阳苹果炭疽叶枯病菌株平均EC_{50}值较低，为0.116 0 mg·L^{-1}。安徽地区，‘金帅’和‘嘎拉’病叶上分离的炭疽叶枯病菌株对戊唑醇的敏感性存在显著差异，‘金帅’和‘乔纳金’病叶上分离的炭疽叶枯病菌株对戊唑醇的敏感性也存在显著差异，‘嘎拉’和‘乔纳金’之间差异不显著（图4-B）。不同地区‘嘎拉’品种上分离的炭疽叶枯病菌株对戊唑醇敏感性差异不显著（图4-C）。



A. 不同地区苹果炭疽叶枯病菌供试菌株对戊唑醇的敏感性差异；B. 安徽砀山同一果园不同苹果品种的炭疽叶枯病菌对戊唑醇敏感性差异；C. 不同地区供试果园嘎拉品种炭疽叶枯病菌对戊唑醇的敏感性差异。差异显著性分析采用 Duncan 氏新复极差法(DMRT)，大写字母表示在 $P < 0.01$ 差异显著。

A. The susceptibility differences to tebuconazole in different areas; B. The sensitivity differences to tebuconazole of different apple varieties in Anhui province; C. The sensitivity differences to tebuconazole of Gala leaves in different areas. Significant differences were analyzed using Duncan's multiple range test (DMRT), different capital letters indicate significant difference at $P < 0.01$.

图4 苹果炭疽叶枯病菌对戊唑醇的敏感性差异

Fig. 4 Sensitive analysis of *Colletotrichum gloeosporioides* against tebuconazole

3 讨 论

笔者通过区分剂量法发现，苹果炭疽叶枯病菌已经对甲基硫菌灵产生高水平抗药性，甲基硫菌灵质量浓度为800 mg·L^{-1}时，对苹果炭疽叶枯病菌的菌丝生长抑制率仅为45.95%。甲基硫菌灵在苹果炭疽叶枯病防治中不能达到预期效果。

对随机挑选的24个甲基硫菌灵高抗菌株的微管蛋白基因进行PCR扩增和序列比对，发现23个菌株的β-tubulin氨基酸序列第198位点由谷氨酸突变为丙氨酸，1个菌株ZG4-7的β-tubulin氨基酸序列第200位点由苯丙氨酸突变为酪氨酸。甲基硫菌灵作为苯并咪唑类杀菌剂，主要抑制生物合成，特异地结合靶标病原菌的β-tubulin微管蛋白，抑制细胞核分裂，而β-tubulin微管蛋白氨基酸序列的突变会导致病原菌对此类杀菌剂产生抗药性[20]。因此苹果炭疽叶枯病菌的微管蛋白第198和200位点产生突变，可能与抗药性相关。

咪唑类杀菌剂咪鲜胺属于甾醇合成抑制剂，通过影响甾醇分子的生物合成，破坏细胞膜的功能[21]。笔者通过区分剂量法和菌丝生长速率法研究发现，苹果炭疽叶枯病菌对咪鲜胺仍然处于较高敏感水平，咪鲜胺对苹果炭疽叶枯病菌的EC_{50}均值为0.011 9 mg·L^{-1}，只有菌株AQ32-9产生低水平抗性，尚未发现高水平抗性菌株。在其他病原真菌，如小麦赤霉病菌和瓜类尖孢镰刀菌，在对咪鲜胺的敏感性测试中没有发现咪鲜胺抗性菌株[22-23]，而水稻恶苗病菌对咪鲜胺的敏感性有明显下降[24]。为延缓苹果炭疽叶枯病菌对咪鲜胺抗性的发展，建议使用不同作用位点的杀菌剂与此类药剂混用或交替使用。

DMIs类杀菌剂戊唑醇，可抑制真菌中麦角甾醇生物合成途径中关键酶CYP51的合成，阻碍麦角甾醇的合成，导致真菌细胞膜结构破坏和细胞死亡，兼具内吸和保护作用，但其作用位点相对单一，可能存在抗药风险[25]。本研究表明，苹果炭疽叶枯病菌已经对戊唑醇产生抗性，20.5%的菌株对戊唑醇已产生高水平抗性。不同地区供试菌株对戊唑醇的敏感性有明显差异，戊唑醇对河北易县菌株的EC_{50}值是河南安阳菌株EC_{50}值的3.4倍，推测抗药性的产生可能与所在地区的农药使用情况相关，建议在苹果炭疽叶枯病的防治中减少戊唑醇的使用，与其他类型杀菌剂轮换使用。

本研究表明，苹果炭疽叶枯病菌在含有5 mg·L^{-1}戊唑醇的PDA平板上生长受到明显抑制，平均抑制率为92.6%，咪鲜胺使用质量浓度为0.5 mg·L^{-1}时，对苹果炭疽叶枯病菌菌丝生长抑制率达到95.02%，但该药剂持效期较短[15]，因此在苹果炭疽叶枯病防治中建议同时使用其他保护性杀菌剂。

4 结 论

我国苹果主产区河南省安阳和郑州、山东省招远和曲阜、河北省易县、陕西省咸阳、安徽省砀山苹果炭疽叶枯病菌已对甲基硫菌灵产生严重抗药性，且测试菌株的微管蛋白第198和200位点产生突变，可能与抗药性相关；苹果炭疽叶枯病菌对戊唑醇产生高水平抗性，发现咪鲜胺低水平抗药性菌株。在苹果炭疽叶枯病菌防治中不建议使用甲基硫菌灵，戊唑醇和咪鲜胺可以应用于苹果炭疽叶枯病的防治中，应注意与其他类型药剂轮换使用。

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