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Inhibitory Effect and Control Efficacy of Picoxystrobin against *Neopestalotiopsis clavispora*, Causing Vine Tea Leaf Blight

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Abstract: Vine tea (*Ampelopsis grossedentata*) is a traditional herb widely consumed in southwestern China that possesses paramount potential for human health. In 2021, the outbreak of typical leaf blight disease was observed in almost all vine tea plantations in Zhangjiajie of Hunan province, resulting in significant economic losses of vine tea production. In this study, we identified *Neopestalotiopsis clavispora* as the causal agent of vine tea leaf blight via its morphological characteristics and molecular identification. The sensitivity distribution of *N. clavispora* isolates to picoxystrobin were determined based on mycelial growth and spore germination inhibition assays. The EC_{50} values for mycelial growth ranged from 0.0062 to 0.0658 μg/mL, with a mean of 0.0282 ± 0.0148 μg/mL. The EC_{50} values for spore germination ranged from 0.0014 to 0.0099 μg/mL, and the mean value was 0.0048 ± 0.0022 μg/mL. Picoxystrobin increased fungal cell membrane permeability, but inhibited fungal ATP biosynthesis. Moreover, picoxystrobin exhibited good in planta control efficacy on vine tea leaves. Three picoxystrobin-resistant mutants were obtained in the current study, but no mutations were detected in the *N. clavispora Cytb* gene. Competitive ability assays showed that the conidium production and pathogenicity of all picoxystrobin-resistant mutants decreased as compared to their progenitors, indicating that picoxystrobin-resistant mutants suffer fitness penalty. These findings provide important evidence for picoxystrobin in vine tea leaf blight management and increase understanding of the resistance mechanism of picoxystrobin against *N. clavispora*.

Keywords: modes of action; *Neopestalotiopsis clavispora*; picoxystrobin; sensitivity distribution; vine tea leaf blight

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1. Introduction

Tea has been an important Chinese traditional beverage for thousands of years. Based on its color, tea is divided into green tea, yellow tea, white tea, oolong, black tea and dark tea. In addition, tea can be classified into fermented, nonfermented or semifermented, according to the different manufacturing processes [1]. To be noted, several herbs, such as *Ampelopsis grossedentata*, also known as vine tea or moyeam, have been used by many ethnic minorities in China to serve as tea-like beverages, which could inhibit various diseases and improve human health [2,3]. Recent studies showed that vine tea is abundant in polyphenols, flavonoids and other natural antioxidants, possessing huge potential for human health [4–6]. Several vine tea extracts have been used as potential natural antioxidants for food applications, which include meat products and soybean oil [7,8]. Significantly, the economic revenue of vine tea during a growing season has surpassed $ 20,000 per ha, further promoting the development of vine tea cultivation in Zhangjiajie.

Before 2010, vine tea was grown naturally in mountainous regions of Zhangjiajie. In recent years, the planting area of vine tea rapidly increased and exceeded 10 thousand ha

in Yongding District, Zhangjiajie, with the economic revenue more than $1.25 billion. In the meantime, a leaf blight of unspecified cause was observed in 90% of vine tea plantations, and no appropriate control strategies were officially developed for disease control, which resulted in direct economic losses of $15 million in 2021. Therefore, it is necessary to characterize the causal agents and screen several chemical fungicides with high efficiency and low toxicity for vine tea leaf blight management.

Quinone outside inhibitors (QoIs) are a group of broad-spectrum fungicides, including pyraclostrobin, azoxystrobin, coumoxystrobin, picoxystrobin, fluoxastrobin, fenaminstrobin, etc. QoIs target the complex III of the fungal respiration pathway and block electron transportation at the Qo center of cytochrome bc1, leading to the reduction of ATP biosynthesis and cell death [9]. Picoxystrobin, a novel member of QoIs, developed by Syngenta, had been extensively used in agriculture owing to its unique distribution properties, including translaminar and systemic (acropetal) movement, diffusion in leaf xylem and molecular redistribution in the air [9,10]. Our preliminary assays showed that picoxystrobin exhibited excellent antifungal effects against the pathogens isolated from vine tea leaf blight, but the modes of action and the in planta control efficacy of picoxystrobin remain unknown. Therefore, the objectives of this study were to (i) isolate and identify the major causal agent of vine tea leaf blight, (ii) determine the picoxystrobin sensitivity distribution of the phytopathogen populations from different counties in vine tea growing regions, (iii) determine the physiological and biological characteristics of the phytopathogen as affected by picoxystrobin, (iv) test the curative and protective effects of picoxystrobin on leaves of vine tea, and (v) evaluate the resistance risk of the phytopathogen to picoxystrobin by comparing the fitness between resistant mutants and sensitive strains. This research will provide important references for the management of vine tea leaf blight in the field.

2. Materials and Methods

2.1. Fungal Isolation and Identification

In 2021 and 2022, a total of 108 symptomatic vine tea leaves with typical leaf blight were collected from 8 vine tea plantations in Zhangjiajie. The diseased leaves were cut into small tissue blocks from the margins of lesions and sterilized in 75% ethanol for 20 s, followed by 3% NaClO for 2 min, then rinsed with sterilized water 3 times. After drying on sterilized filter paper, the tissues were placed onto water agar (WA) plates amended with 30 μg/mL streptomycin sulphate. After incubation for 4 days at 28 °C, 56 isolates with similar morphological characteristics were purified by transferring a single mycelial tip to a new potato dextrose agar (PDA) plate for further study [11–13]. Furthermore, 3 isolates were arbitrarily selected and inoculated on vine tea leaves, following the procedures of Koch's postulates, and photos were taken after 7 days of incubation.

Among the 56 isolates, 5 isolates were arbitrarily chosen for DNA extraction and PCR amplification. The partial sequences of internal transcribed spacers (*ITS*), β-tubulin (*TUB*) and translation elongation factor 1 (*TEF1*) were amplified with primers, as described previously [14], and for DNA sequencing (Sangon, China). The sequences were subjected to a BLAST search in NCBI GenBank, and the phylogenetic tree was generated with the neighbor-joining method by MEGA5, as described previously [11].

2.2. DNA Extraction and PCR Assays

The fungal DNA was extracted via a Phire Plant Direct PCR Kit (Thermo Scientific, Waltham, MA, USA, F130WH). Briefly, 10 μL of dilution buffer was added into a sterilized 1.5 mL tube, then a few mycelia from the edge of a 3-day-old colony were scraped by sterilized toothpick and mixed with dilution buffer, followed by a 15 s vibration. After a short centrifugation, the buffer was used for PCR reaction. The PCR was performed with 2 × Taq Plus Master Mix II (Dye Plus) (Vazyme Biotech, Nanjing, China) in a 25 μL reaction mixture. The PCR was conducted via a MasterCycler^{pro} thermal cycler (BioRad, Hercules, CA, USA), as described previously [14]. The cycling conditions were as follows: an initial denaturation step at 95 °C for 3 min; followed by 35 cycles, each consisting of 95 °C for 15 s

denaturing; annealing at 56 °C for 20 s; and 72 °C for 40 s, with a final extension at 72 °C for 5 min.

2.3. Fungicide and Media

Technical-grade picoxystrobin (98%, Shandong Weifang Rainbow Chemical Co., Ltd.; Weifang, China) was dissolved in methanol to 10 mg/mL and stored at 4 °C as stock solution. For the sensitivity test, stock solution was diluted to a series of concentrations and added into alkyl ester agar (AEA) media. Meanwhile, salicylhydroxamic acid (SHAM) was added to AEA media at 50 μg/mL to suppress the alternative oxidase pathway.

WA media contained 5 g of agar powder in 1 L of deionized water. PDA media contained 20 g of dextrose, 200 g of sliced, unpeeled potatoes and 20 g of agar powder in 1 L of deionized water. AEA media contained 6 g of $NaNO_3$, 5 g of yeast extract, 1.5 g of KH_2PO_4, 0.51 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 20 mL of glycerin and 16 g of agar powder in 1 L of deionized water. Yeast extract peptone dextrose (YEPD) media contained 3 g of yeast extract, 10 g of peptone and 20 g of dextrose in 1 L of deionized water.

2.4. Sensitivity of Mycelial Growth of Neopestalotiopsis clavispora to Picoxystrobin

The picoxystrobin sensitivity of *N. clavispora* was performed with 56 strains, using the mycelial growth inhibition method, as described previously [15–18]. Mycelial plugs (4 mm diameter) were taken from 5-day-old colonies and transferred onto AEA plates amended with 0, 0.015625, 0.03125, 0.0625, 0.125, 0.25 and 0.5 μg/mL of picoxystrobin each. After incubation for 7 days at 28 °C, photos were taken, and the colony diameters were measured in 2 perpendicular directions for the calculation of the median effective concentration (EC_{50}) according to the mycelial growth inhibition rates. All concentrations had three replicates, and the experiments were performed twice.

2.5. Sensitivity of Conidium Germination of N. clavispora to Picoxystrobin

The picoxystrobin sensitivity of *N. clavispora* was performed with 19 strains, using the conidium germination inhibition method, as described previously [12,18]. All selected isolates were grown on a PDA plate at 28 °C for 21 days in the dark. The conidia produced on the PDA plate were washed with 3 mL sterilized water, filtered with 3-layer lens papers and centrifuged for 2 min at 8000 rpm. The conidia were resuspended with sterilized water and adjusted to 1×10^5 conidia/mL using a hemocytometer. Then, 100 μL of conidia suspension was spread on WA plates amended with 0, 0.005, 0.01, 0.02 and 0.04 μg/mL of picoxystrobin each. The germination rate was observed with a microscope after incubation at 28 °C for 12 h in the dark. All concentrations had two replicates, and the experiments were performed twice.

2.6. Effect of Picoxystrobin on Pycnidium Formation of N. clavispora

N. clavispora strains ZJJ5, ZJJ9 and ZJJ12 were randomly chosen for a pycnidium formation assay. Mycelial plugs (4 mm diameter) were taken from a 5-day-old colony and transferred onto PDA plates amended with 0, 0.015625, 0.03125, 0.0625, 0.125, 0.25 and 0.5 μg/mL of picoxystrobin each. All plates were incubated at 28 °C for 21 days with 4 replicates, then photos were taken and the number of pycnidium (black particles) on each plate was counted. All treatments had four replicates, and the experiments were performed twice.

2.7. Cell Membrane Permeability as Affected by Picoxystrobin

N. clavispora strains ZJJ5, ZJJ9 and ZJJ12 were selected to determine the cell membrane permeability in the presence of picoxystrobin, according to previous studies [17,18], with minor modification. A total of 15 mycelial plugs were taken from 5-day-old colonies and transferred into 100 mL liquid AEA media. After incubating for 48 h, the final concentrations of picoxystrobin and SHAM in media were added to be 0.56 μg/mL and 50 μg/mL, respectively. After another 24 h of incubation, mycelia were harvested with a filter cloth.

Overall, 0.15 g of mycelia were suspended in 15 mL deionized water, and the conductivity of the mycelia was measured at different time points with the DDS-307A conductivity meter (Leici, Shanghai, China). After 180 min, the suspensions were boiled for 10 min, and the final conductivity was determined. The experiments were performed twice, and all treatments had three replicates. The relative conductivity was calculated as the following equation:

$$\text{Relative conductivity } (\%) = \frac{\text{real} - \text{time conductivity}}{\text{final conductivity}} \times 100 \quad (1)$$

2.8. ATP Production Assays (1)

Three *N. clavispora* strains, ZJJ5, ZJJ9 and ZJJ12, were used to determine the ATP biosynthesis in the presence of picoxystrobin. In total, 15 mycelial plugs were taken from 5-day-old colonies and transferred into 100 mL liquid AEA media. After incubating for 2 days, the final concentrations of picoxystrobin and SHAM in media were added to be 0.2 μg/mL and 50 μg/mL, respectively. After another 24 h of incubation, the mycelia were harvested with a filter cloth and grinded with liquid nitrogen. The ATP production was assayed using an ATP Assay Kit (Solarbio, Beijing, China, BC0300). All treatments had three replicates, and the experiments were performed twice.

2.9. Control Efficacy of Picoxystrobin on Vine Tea

The protective and curative efficacy of picoxystrobin on vine tea against *N. clavispora* were performed according to recent studies [18,19], with several modifications. The leaves of vine tea were disinfected in 0.5% NaClO for 20 s, then washed with sterilized water twice and air-dried naturally. Picoxystrobin stock solution was diluted to 2 and 4 μg/mL with 0.1% tween 20. For the protective activity test, picoxystrobin or water was sprayed onto leaves of vine tea. After incubation at 28 °C for 1 day, 10 leaves were stabbed with a sterilized needle and inoculated with 2.5 μL conidial suspension (5×10^5–1×10^6). After incubated for another 4 days, photos were taken, and the diameters of the lesions were measured. For the curative activity test, 10 leaves of vine tea were inoculated with 2.5 μL conidial suspension, as described above, prior to picoxystrobin or water spraying. After incubation for 1 day at 28 °C, the leaves of vine tea were sprayed with picoxystrobin or water. Photos were taken, and the diameters of the lesions were measured after inoculation for another 4 days. The control efficacy was calculated according to the previous method [16,18]. Three *N. clavispora* strains, ZJJ5, ZJJ9 and ZJJ12 were used for inoculation. The experiments were performed twice, and each experiment had 10 vine tea leaves.

2.10. In Vitro Induction of Picoxystrobin-Resistant Mutants

In order to obtain picoxystrobin-resistant mutants, mycelial plugs (4 mm in diameter) taken from 5-day sensitive strains were placed on AEA plates (a total of 200 mycelium plugs of each isolate, 10 mycelial plugs for each plate) amended with 8 μg/mL picoxystrobin, and all plates were exposed to UV light for 15 s and then incubated at 28 °C for 21 days in the dark. Putative picoxystrobin-resistant mutants were isolated from colonies and placed on AEA plates amended with 8 μg/mL picoxystrobin and picoxystrobin-free PDA plates. The mutants that grew on the AEA plates amended with 8 μg/mL picoxystrobin were identified as picoxystrobin-resistant mutants.

2.11. Resistance Stability Assays

To evaluate whether the resistance level of picoxystrobin-resistant mutants was stable or not, three laboratory-induced picoxystrobin-resistant mutants were transferred one time, ten times and fifteen times on picoxystrobin-free PDA plates. The picoxystrobin-resistant mutants were allowed to grow for 4 days at 28 °C after each transferring. The EC_{50} values of all mutants were measured as above described after one, ten and fifteen transfer times. The three EC_{50} values of each mutant were compared. The experiments were performed

twice, and all concentrations had three replicates. The resistance level of each mutant was presented by the resistance factor (RF), and the formulas are depicted as follows:

$$\text{Resistance factor} = \frac{\text{The } EC_{50} \text{ of the picoxystrobin} - \text{resistant mutant}}{\text{The } EC_{50} \text{ of the picoxystrobin} - \text{sensitive progenitor}} \quad (2)$$

2.12. Sequencing Analysis of N. clavispora Cytb Gene

With the shortage of *Cytb* gene nucleotide sequences of *N. clavispora* in NCBI databases, we used the *Cytb* gene of *Pestalotiopsis longiseta* (accession number FJ811964) as the reference sequence for the primers design. Primers *NcCytb-F* (5′-TGGATGATTAGTTCGTTAC-3′) and *NcCytb-R* (5′-ATATCTTGTCCAATTCATGG-3′) were designed to amplify the partial *Cytb* gene fragment (261 bp) from picoxystrobin-sensitive strain ZJJ5 and the picoxystrobin-resistant mutants ZJJ5-K2, ZJJ5-K3 and ZJJ5-K5. The cycling conditions were as follows: an initial denaturation step at 95 °C for 3 min; followed by 35 cycles, each consisting of 95 °C for 15 s denaturing; annealing at 50 °C for 20 s; and 72 °C for 40 s, with a final extension at 72 °C for 5 min. The PCR products were cleaned with a Gel DNA extraction kit (Vazyme, Nanjing, China, DC301-01) and sequenced by Sangon Co., Ltd. (Shanghai, China). Further analyses of sequences were performed with the BioEdit Software (Version 7.0.9.0).

2.13. Cross-Resistance Assays

Three laboratory-induced picoxystrobin-resistant mutants were used to determine the pattern of cross-resistance, as described previously [20,21]. Mycelial plugs taken from the margin of 5-day-old colonies were placed on AEA plates amended with various gradients of concentration of picoxystrobin, pyraclostrobin and azoxystrobin, or placed on PDA plates amended with various gradients of concentration of difenoconazole, carbendazim and phenamacril (Supplementary Table S1). After incubation at 28 °C for 5 days, the colony diameters were measured, and the EC_{50} values were calculated via DPS software, as described previously [22,23]. The experiments were performed three times, and all concentrations had three replicates.

2.14. Fitness Tests

Three picoxystrobin-resistant mutants (ZJJ5-K2, ZJJ5-K3 and ZJJ5-K5) and their parental strain (ZJJ5) were used to evaluate the potential competition between picoxystrobin-resistant and picoxystrobin-sensitive strains. All strains were incubated at 28 °C for 5 days on PDA plates, and the colony diameters were measured. For biomass assays, 10 mycelial plugs of each strain were taken from 5-day-old colonies and transferred into 100 mL liquid YEPD media. After incubation at 28 °C, 175 rpm for 3 days, mycelia were harvested, air-dried and weighed. The conidial production of all strains was maintained and conducted, as described above. The differences in pathogenicity between picoxystrobin-sensitive strains ZJJ5 and the three picoxystrobin-resistant mutants were compared on vine tea leaves. A total of 10 leaves of vine tea were pierced with a sterilized needle and inoculated with mycelial plugs (4 mm diameter) taken from 5-day-old colonies on PDA plates. The leaves were incubated at 28 °C for 7 days, and the lesion diameter on each leaf was measured. The assays were performed twice with 10 leaves.

2.15. Data Analysis

Data on vegetative growth, conidia production, ATP production, protective and curative activity, relative conductivity and the EC_{50} value of *N. clavispora* isolates were subjected to analysis of variance. Fisher's LSD test was used to determine the differences ($p < 0.05$) among each treatment. All statistical analyses were performed with Data Processing System (DPS) software (Version 19.05).

3. Results

3.1. Morphological and Molecular Characteristics of Fungal Isolates

In the current study, 56 isolates with a similar colony morphology were obtained from symptomatic vine tea leaves with typical leaf blight (Figure 1A). The colony showed a smooth edge, white with a concentric circle, and dense aerial mycelia (Figure 1B). After a 14-day incubation, pycnidia (black fruiting bodies) formed on the PDA plates and deeply embedded in the medium (Figure 1B). The isolates produced five-celled conidia, which were fusiform, with two colorless terminal cells and three-colored median cells, as well as one hyaline basal appendage and two to three hyaline apical appendages arising from the apex (Figure 1B). Furthermore, the disease symptoms were obvious after 7 days of incubation (Figure 1C), and the original pathogen was re-isolated from the diseased vine tea leaves.

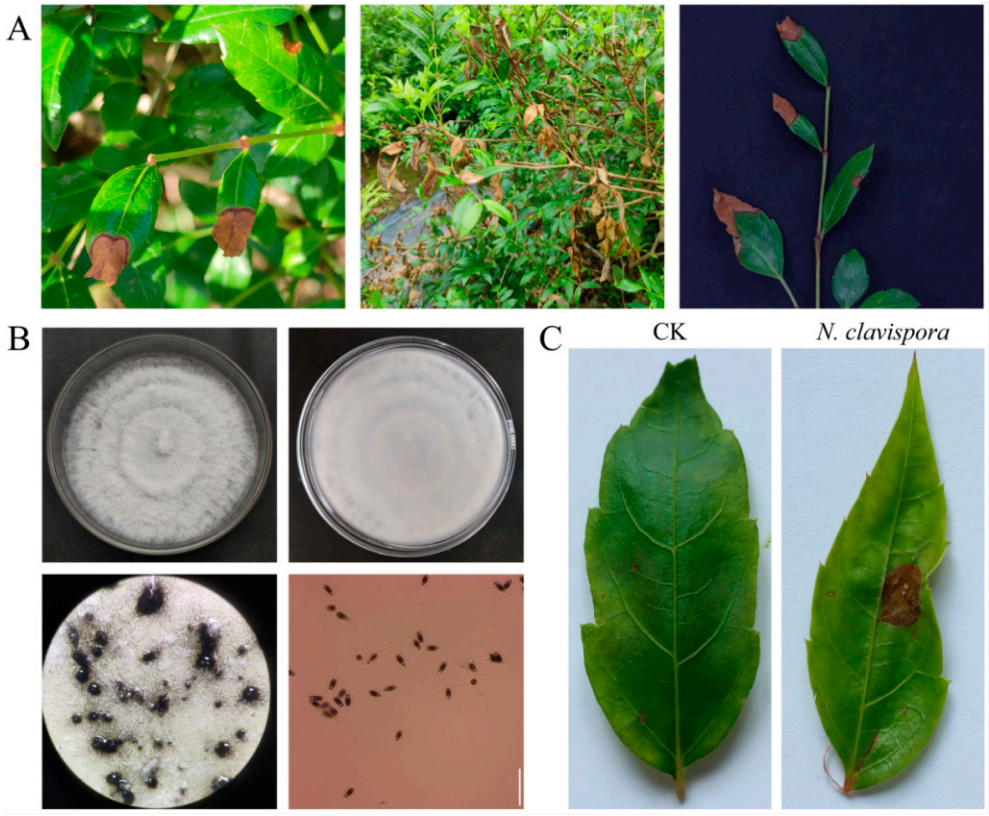

Figure 1. Isolation of *N. clavispora*. (**A**) Typical symptoms of vine tea leaf blight in the field. (**B**) Colonies, pycnidia and conidia morphology of *N. clavispora*. Bar = 50 μm. (**C**) Disease symptom of *N. clavispora* on vine tea leaves in greenhouse.

The combined dataset of the *ITS*, *TUB* and *TEF1* sequences was used for phylogenetic analyses conducted by neighbor-joining. The accession number of all five isolates are provided in Supplementary Table S2. According to the results, all five isolates (ZZJ5, ZZJ8, ZZJ10, ZZJ17 and ZZJ-YK) were clustered with *N. clavispora* isolates LA-01 and PS110 (Figure 2).

3.2. Sensitivity Distribution of N. clavispora to Picoxystrobin Based on Mycelial Growth Inhibition

As the results showed, the inhibitory rate of picoxystrobin increased with the increase of the dosage (Figure 3A). In addition, all isolates were sensitive to picoxystrobin, the EC_{50} values of the 56 isolates ranged from 0.0062 to 0.0658 μg/mL, and the mean value was 0.0242 ± 0.0127 μg/mL (Figure 3B). These results indicated that picoxystrobin had a strong inhibitory activity on the mycelial growth of *N. clavispora* in vitro.

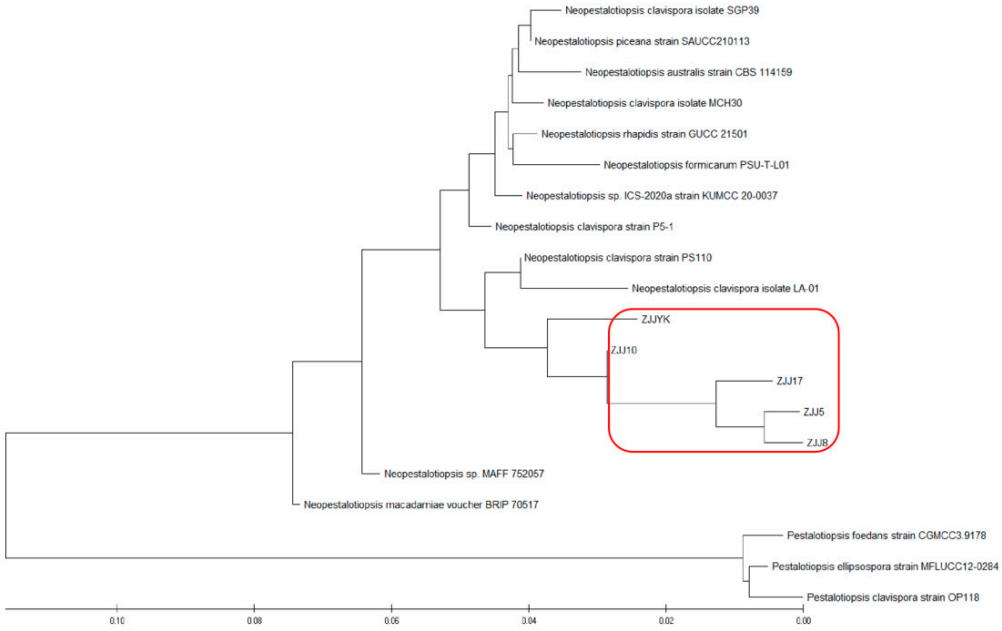

Figure 2. Phylogenetic tree based on the concatenated sequences of *ITS*, *TUB* and *TEF1-α* genes of 5 selected isolates, 12 neopestalotiopsis isolates and 3 pestalotiopsis isolates from NCBI database. The tree was produced using the neighbor-joining analysis. *N. clavispora* isolates (ZJJ5, ZJJ8, ZJJ10, ZJJ17 and ZJJYK) are marked with red box. Bar: the estimated nucleotide substitutions per site are 0.02.

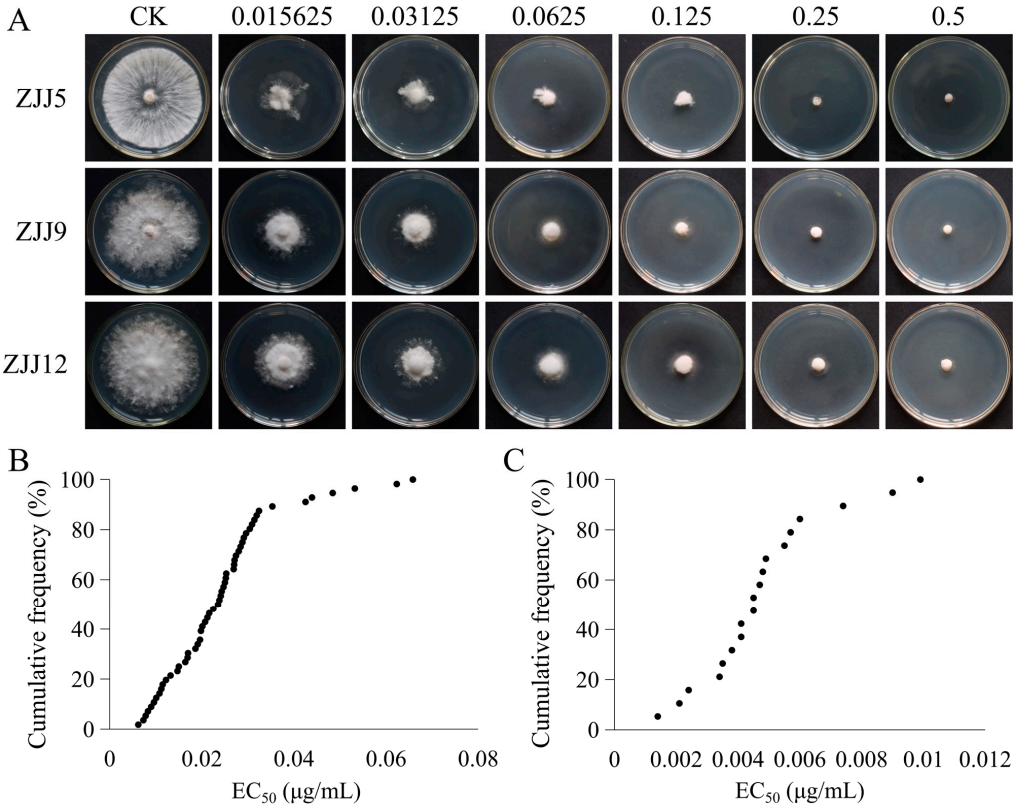

Figure 3. Effect of picoxystrobin against *N. clavispora* in vitro. (**A**) Inhibitory effect of picoxystrobin on mycelial growth of *N. clavispora* strains (ZJJ5, ZJJ9 and ZJJ12) on AEA plates. Photos were taken after growth for 6 days at 28 °C. (**B**) Sensitivity distribution of 56 *N. clavispora* isolates to picoxystrobin based on mycelial growth inhibition. (**C**) Sensitivity distribution of 19 *N. clavispora* isolates to picoxystrobin based on conidium germination inhibition.

3.3. Sensitivity Distribution of N. clavispora to Picoxystrobin Based on Conidium Germination Inhibition

As the results showed, the inhibitory rate of picoxystrobin increased with the increase of the dosage. In addition, all isolates were sensitive to picoxystrobin, the EC_{50} values of picoxystrobin ranged from 0.0014 to 0.0099 μg/mL, and the mean value was 0.0048 ± 0.0022 μg/mL (Figure 3C), indicating that picoxystrobin had excellent inhibitory activity on the conidium germination of *N. clavispora* in vitro.

3.4. Effect of Picoxystrobin on Mycelial Morphology of N. clavispora

The hyphae of *N. clavispora* became sparser and exhibited decreased apical branching when treated with 0.2 μg/mL picoxystrobin, as compared to the non-picoxystrobin-treated group, which was normal (Figure 4).

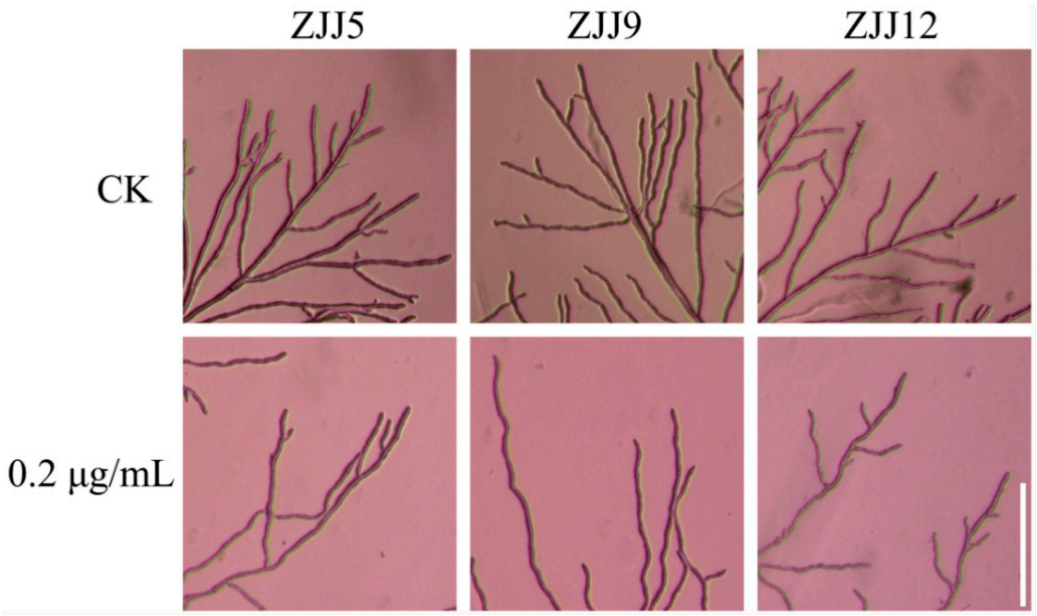

Figure 4. Effect of 0.2 μg/mL picoxystrobin on mycelia morphology of *N. clavispora* strains (ZJJ5, ZJJ9 and ZJJ12). All strains showed less apical branching when treated with 0.2 μg/mL picoxystrobin as compared to control group. Bar = 100 μm.

3.5. Effect of Picoxystrobin on Pycnidium Formation of N. clavispora

The numbers of *N. clavispora* pycnidia significantly decreased when treated with 0.015625, 0.03125, 0.0625, 0.125 and 0.25 μg/mL picoxystrobin, as compared to the non-picoxystrobin-treated group (Figure 5). Furthermore, the conidium production significantly decreased in the presence of picoxystrobin These results suggested that picoxystrobin disrupted the pycnidium formation of *N. clavispora*.

3.6. Effect of Picoxystrobin on Cell Membrane Permeability of N. clavispora

The results showed that the relative conductivity significantly increased after treatment with 0.2 μg/mL picoxystrobin when compared to the non-picoxystrobin group (Supplementary Figure S1), indicating that picoxystrobin disrupted the cell membrane permeability in *N. clavispora*.

3.7. Picoxystrobin Inhibited ATP Biosynthesis in N. clavispora

In the presence of 0.2 μg/mL picoxystrobin, the ATP production decreased by a factor of 29.6%, 33.4% and 26.7% in the 3 *N. clavispora* strains ZJJ5, ZJJ9 and ZJJ12, respectively, as compared to the control groups (Figure 6), demonstrating that picoxystrobin inhibited ATP biosynthesis in *N. clavispora*.

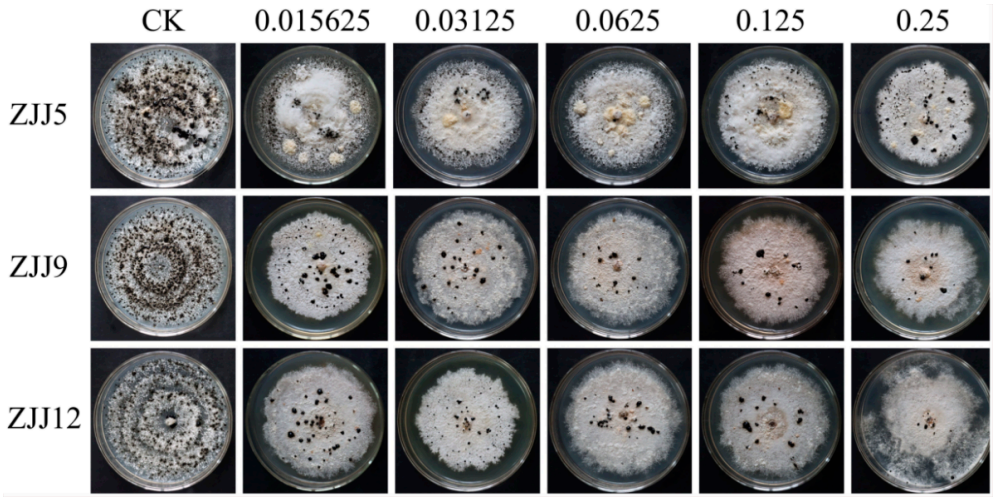

Figure 5. Effect of different concentrations of picoxystrobin on pycnidia formation of *N. clavispora* strains (ZJJ5, ZJJ9 and ZJJ12). The colonies were grown at 28 °C for 4 weeks, then the photos were taken.

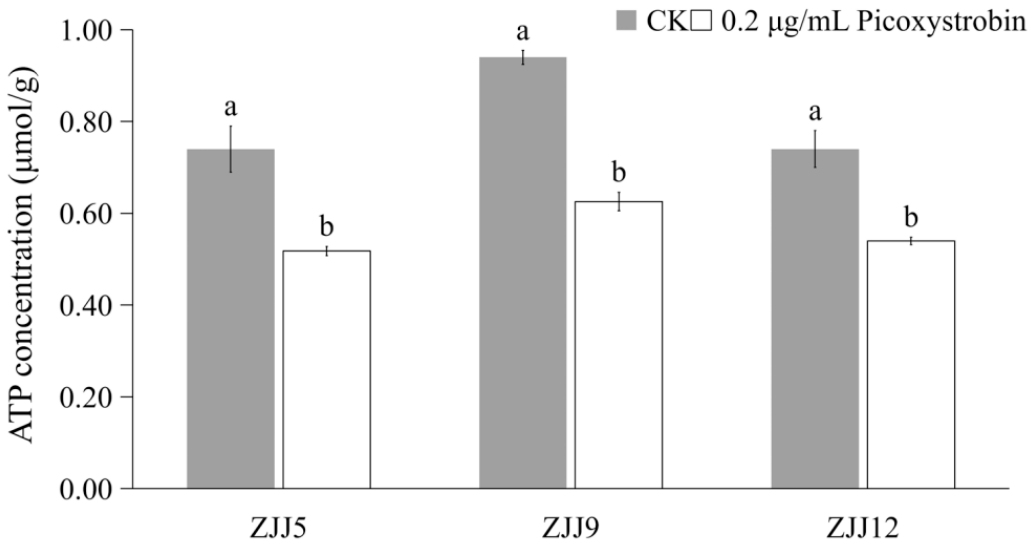

Figure 6. Effect of 0.2 μg/mL picoxystrobin on ATP biosynthesis of *N. clavispora* strains (ZJJ5, ZJJ9 and ZJJ12). Bars denote standard errors from three repeated experiments. Values on the bars followed by the same letter are not significantly different at $p = 0.05$ according to Fisher's least significant difference (LSD) test.

3.8. In Planta Control Efficacy of Picoxystrobin against N. clavispora

In the current study, picoxystrobin exhibited outstanding antifungal activity in the mycelial growth and conidium germination of *N. clavispora*. Therefore, to confirm the efficacy of picoxystrobin in controlling vine tea leaf blight, in planta experiments of picoxystrobin were performed on vine tea leaves. As the results showed, picoxystrobin markedly decreased the lesion diameter of inoculated leaves in both the curative activity and protective activity assays. For the protective activity assays, picoxystrobin showed 70.1 and 89.9% control efficacies for 2 and 4 μg/mL, respectively. However, the control efficacies of picoxystrobin for the curative activity assays were 59.6 and 79.7% for 2 and 4 μg/mL, respectively (Figure 7). These results suggested that picoxystrobin exhibited both good protective and curative activities, and that the protective activity was superior to the curative activity at the same dose of fungicide.

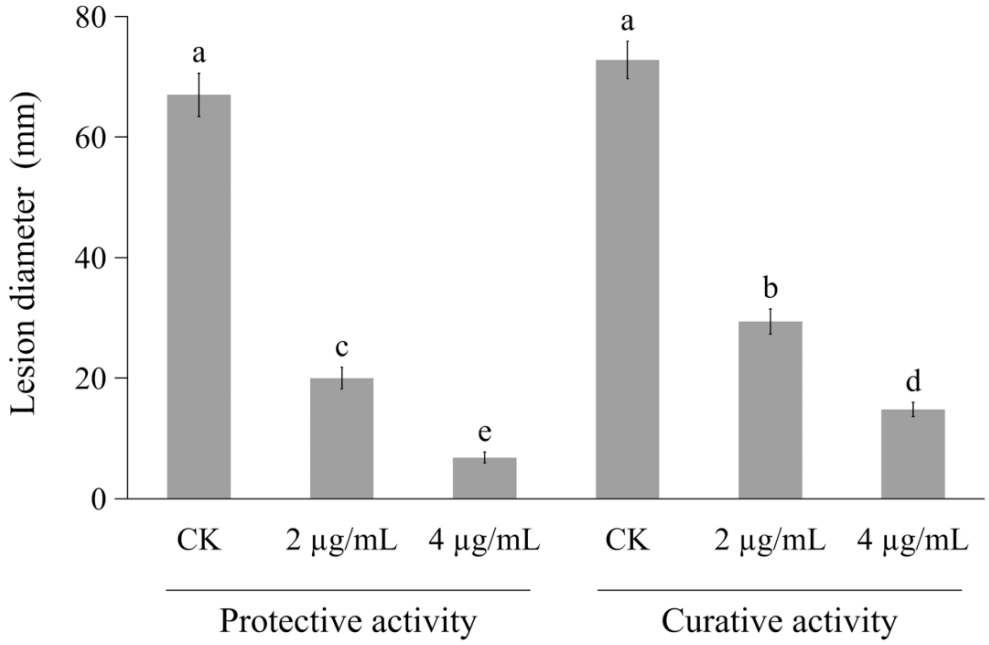

Figure 7. Lesion diameter for protective and curative activity of picoxystrobin. Bars denote standard errors from two repeated experiments. Values on the bars followed by the same letter are not significantly different at $p = 0.05$ according to Fisher's least significant difference (LSD) test.

3.9. Cross-Resistance between Picoxystrobin and Other Fungicides in N. clavispora

Our results showed that three picoxystrobin-resistant mutants were still resistant to picoxystrobin after their fifteen transfers on picoxystrobin-free PDA plates according to their RF values (Table 1, Supplementary Table S3). When we compared the EC_{50} values between the picoxystrobin-sensitive strain ZJJ5 and the three picoxystrobin-resistant mutants, we found that the high-picoxystrobin-resistant mutant ZJJ5-K3 was also resistant to QoIs pyraclostrobin and azoxystrobin via Spearman's rank correlation coefficients analysis, and the correlation coefficients were 0.949 and 0.775, respectively, while the picoxystrobin-resistant mutants ZJJ5-K2 and ZJJ5-K5 were sensitive to pyraclostrobin and azoxystrobin as compared with ZJJ5. To be noted, the EC_{50} values for difenoconazole, carbendazim and phenamacril did not differ among the three picoxystrobin-resistant mutants and the picoxystrobin-sensitive strain ZJJ-5 (Table 1), demonstrating that no cross-resistance was observed between picoxystrobin and difenoconazole, carbendazim or phenamacril in *N. clavispora*.

3.10. Analysis of Cytb Gene in Picoxystrobin-Resistant Mutants

Sequencing alignment analyses showed that the picoxystrobin-resistant mutants (ZJJ-K2, ZJJ-K3 and ZJJ-K5) had no mutations at codon 129 (TTC, phenylalanine) or 143 (GGT, glycine) in the *Cytb* gene as compared to the picoxystrobin-sensitive strain ZJJ5 (OQ325327) (Supplementary Figure S2), suggesting that the mechanism responsible for picoxystrobin resistance in *N. clavispora* is not associated with typical mutations in the *Cytb* gene.

Table 1. Sensitivities of *Neopestalotiopsis clavispora* isolates to picoxystrobin and other fungicides.

Isolates	Picoxystrobin		Azoxystrobin		Pyraclostrobin		Difenoconazole		Phenamacril		Carbendazim	
	EC_{50} (μg/mL) [a]	RF [b]	EC_{50} (μg/mL) [a]	RF [b]	EC_{50} (μg/mL) [a]	RF [b]	EC_{50} (μg/mL) [a]	RF [b]	EC_{50} (μg/mL) [a]	RF [b]	EC_{50} (μg/mL) [a]	RF [b]
ZJJ5	0.025 ± 0.0042 d		0.014 ± 0.0021 c		0.006 ± 0.0012 b		1.00 ± 0.084 bc		0.97 ± 0.078 b		0.075 ± 0.0063 b	
ZJJ5-K2	0.32 ± 0.014 c	12.80	0.014 ± 0.0023 c	1.00	0.006 ± 0.0014 b	1.00	0.97 ± 0.074 c	0.97	1.05 ± 0.085 b	1.08	0.072 ± 0.0052 b	0.96
ZJJ5-K3	3.22 ± 0.31 a	128.80	1.03 ± 0.092 a	73.57	0.93 ± 0.081 a	155.00	3.61 ± 0.30 a	3.61	2.05 ± 0.10 a	2.11	0.13 ± 0.0084 a	1.73
ZJJ5-K5	0.99 ± 0.087 b	39.60	0.036 ± 0.0063 b	2.57	0.006 ± 0.0011 b	1.00	1.16 ± 0.090 b	1.16	0.96 ± 0.081 b	0.99	0.073 ± 0.0063 b	0.97

[a] Means and standard deviations (SDs) were calculated from three independent assays. Different letters indicate significant differences according to the Fisher's LSD test at $p = 0.05$. [b] RF (resistance factor) = EC_{50} value (resistant mutants)/EC_{50} value (wild-type isolate).

3.11. Competitive Abilities of Picoxystrobin-Resistant Mutants of N. clavispora

Although the mycelial growth of the picoxystrobin-resistant mutants ZJJ5-K2 and ZJJ5-K5 showed no difference when compared to their progenitor ZJJ5, the colony diameter of the picoxystrobin-high-resistant mutant ZJJ5-K3 significantly decreased. In addition, the mycelial dry weight of the picoxystrobin-resistant mutants ZJJ5-K5 and ZJJ5-K3 significantly decreased as compared to their parental strain ZJJ5 when cultured in YEPD medium for 3 days. When incubated on PDA plates at 28 °C for 21 days, the conidia production of all mutants significantly decreased as compared to their parental strain ZJJ5. Furthermore, we evaluated the pathogenicity of the picoxystrobin-resistant mutants on vine tea leaves. As the results showed, all the mutants produced smaller lesion areas when compared to their parental strain ZJJ5 (Table 2), demonstrating that the pathogenicity of the picoxystrobin-resistant mutants significantly decreased as compared to the picoxystrobin-sensitive strains in *N. clavispora*.

Table 2. Fitness of picoxystrobin-resistant mutants of *Neopestalotiopsis clavispora*.

Strains	Colony Diameter (cm) *	Dry Weight (g) *	Conidia Concentration ($\times 10^5$/mL) *	Lesion Diameter (mm) *
ZJJ5	6.79 ± 0.12 a	0.66 ± 0.11 a	9.70 ± 0.53 a	13.5 ± 1.4 a
ZJJ5-K2	6.63 ± 0.09 a	0.48 ± 0.10 ab	8.53 ± 0.21 b	7.0 ± 0.6 b
ZJJ5-K3	3.81 ± 0.1 b	0.07 ± 0.02 c	3.80 ± 0.36 c	4.7 ± 0.5 c
ZJJ5-K5	6.68 ± 0.13 a	0.40 ± 0.06 b	7.93 ± 0.31 b	6.3 ± 0.8 b

* Means and standard deviations (SDs) were calculated from three independent experiments. Values in the table followed by the same letter along a column are not significantly different at $p = 0.05$ according to Fisher's least significant difference (LSD) test.

4. Discussion

The fast-growing tea market and increasing desire for healthier foods, such as tea and tea-derived products, have pushed the tea industry to improve its product quality, safety and variety [24]. As one of the traditional herbs in China, vine tea has been widely consumed for years because of its potential health benefits [25,26]. In the meantime, a leaf blight was found in almost all vine tea plantations in Zhangjiajie, causing direct economic losses of approximately $15 million in 2021. In the current study, we identified *N. clavispora* as the causal agent of vine tea leaf blight, according to morphological and molecular characterization assays. In addition, artificial inoculation assays exhibited similar disease symptoms on vine tea leaves, further demonstrating that *N. clavispora* is the causal agent of vine tea leaf blight. The genus *Pestalotiopsis* is a heterogeneous group of fungi containing more than 220 species, which are differentiated basically by their conidial characteristics (CABI Bioscience database). As a member of the pestalotioid species, *N. clavispora* was found on various hosts worldwide, including *Kadsura coccinea*, strawberry, etc., posing a threat to agricultural production by causing reduced plant quality, yield loss and disruption of production schedules [27,28]. However, less information on the chemical control strategy for *N. clavispora* is available.

Picoxystrobin is a new QoI with systemic and osmotic activity [9], which has been applied in various crops' disease management, including economic crops, cereal grains (except rice) and fruits [10,29] (http://www.chinapesticide.org.cn/) (accessed on 4 May 2023). In the current study, the picoxystrobin sensitivity of *N. clavispora* was determined based on mycelial growth and conidium germination inhibition. The EC_{50} values of 56 *N. clavispora* isolates ranged from 0.0062 to 0.0658 μg/mL, with a mean of 0.0242 μg/mL for mycelial growth inhibition, and the EC_{50} values of 19 randomly selected isolates ranged from 0.0014 to 0.0099 μg/mL, with a mean of 0.0048 μg/mL for conidium germination inhibition. Our research found that the antifungal activity of picoxystrobin against *N. clavispora* was higher than the QoI fungicide azoxystrobin and other widely used fungicides with different modes of action (triazoles, SDHIs and carbendazim). Taken together, these data demonstrated that picoxystrobin has an excellent effect on both the mycelial growth

and conidium germination of *N. clavispora*, which possesses great potential for further application in the control of vine tea leaf blight in the field.

Mitochondrion is one of the core organelles that regulate cell fate decisions via their biological functions in cell growth, metabolism and cell death. Among various metabolic functions, ATP biosynthesis that depends on oxidative phosphorylation and the tricarboxylic acid cycle is the most important [30,31]. As a classical QoI, picoxystrobin inhibits fungal respiration by blocking the electron transportation of cytochrome bc1 complex [9]. In a previous study, Duan and colleagues found that ATP biosynthesis was significantly inhibited by six QoIs in *F. graminearum* [32]. In the current study, the inhibitory ratio of conidium germination was approximately 90% in the presence of 0.04 μg/mL picoxystrobin. Moreover, the intracellular ATP biosynthesis of *N. clavispora* isolates was significantly suppressed when treated with 0.2 μg/mL picoxystrobin. These data indicated that picoxystrobin could lead to the reduction of ATP production in *N. clavispora*, and may attribute to disease control in the field.

To explore the potential application of picoxystrobin in controlling vine tea leaf blight, artificial inoculation assays were carried out in the current study. Our results showed that picoxystrobin had both curative and protective effects on leaves of vine tea. The control effect of picoxystrobin increased with the increase of the fungicide concentration, and the protective effect was better than the curative effect. According to our survey, most of the vine tea growers are accustomed to applying fungicides after diseases emerge in the field, which decreases the control efficacy and results in huge economic losses in agricultural production. Our results and several previous publications suggested that the time point of fungicide application is critical for disease management, and the protective effect is always better than the curative effect with the same fungicides doses [18,33,34]. Taken together, the above results indicated that picoxystrobin had a good potential to control vine tea leaf blight caused by *N. clavispora*.

Fungicide resistance is a common phenomenon embodied in the natural process of the evolution of biological systems. In the meantime, it is a major concern that threatens sustainable disease management in agricultural production [35–39]. Therefore, we evaluated the potential resistant risk of *N. clavispora* to picoxystrobin in the current study. The results showed that all picoxystrobin-resistant mutants were still resistant to picoxystrobin after their fifteen transfers on picoxystrobin-free PDA plates. DNA sequencing showed that no mutations were detected at codon 129 or 143 in the *Cytb* of picoxystrobin-resistant mutants, indicating that the mechanism responsible for picoxystrobin resistance in *N. clavispora* is not associated with typical mutations in the *Cytb* gene. In addition, we could not generate *Cytb* gene point mutation mutants from *N. clavispora* due to the shortage of nucleotide sequences in NCBI and other databases. Significantly, additional picoxystrobin-resistant mutations and mechanisms have been claimed in several phytopathogens [40,41]. It is possible that mutations existed in alternative respiration and efflux transporters, which had been reported previously [42,43]. Intriguingly, picoxystrobin-high-resistant mutant ZJJ5-K3 showed cross-resistance between picoxystrobin and other QoIs (azoxystrobin and pyraclostrobin), while no cross-resistance was found between picoxystrobin and other QoIs, as well as fungicides with different modes of action in the picoxystrobin-resistant mutants ZJJ5-K2 and ZJJ5-K5. These data indicated that the combination of picoxystrobin and fungicides with different modes of action could minimize its resistance risk in vine tea leaf blight management.

The fitness of resistant fungal isolates is a vital factor in evaluating the development of the fungicide-resistant population in the field. Previous studies found that the carbendazim-resistant population of *Fusarium graminearum* exhibited no obvious defects in mycelial growth and stress response, but possessed increased deoxynivalenol (DON) production and elevated virulence on wheat head [44–46]. Similar results were also found in *Botrytis cinerea*, *Sclerotinia sclerotiorum* and several important plant pathogens [21,36,47,48]. Previous research showed that resistant individuals with the G143A substitution of *Cytb* do not suffer from a significant fitness penalty [49]. Although no significant defect was found

in colony morphology, mycelial growth and biomass between picoxystrobin-sensitive strain ZJJ5, picoxystrobin-resistant mutants ZJJ5-K2 and ZJJ5-K5, all picoxystrobin-resistant mutants exhibited severe defects in conidium production and pathogenicity in the current studies. Notably, these biological defects were more serious in picoxystrobin-high-resistant mutant ZJJ5-K3. Taken together, we suggested that the picoxystrobin-resistant populations could not develop quickly due to their low fitness as compared to picoxystrobin-sensitive populations in the field, and the resistance risk of *N. clavispora* to picoxystrobin is medium to high in the laboratory.

5. Conclusions

In the current study, *N. clavispora* was identified as the causal agent of vine tea leaf blight, according to fungal colony morphology and molecular identification assays. Picoxystrobin not only exhibited excellent antifungal activity, but good in planta control efficacy against *N. clavispora*. Picoxystrobin blocks the electron transportation of cytochrome bc1 complex, leading to the shortage of intracellular ATP production, thereby causing the defects in energy metabolism, conidium germination and plant infection of *N. clavispora*. Although our results found that cross-resistance was observed between picoxystrobin and other QoIs, no cross-resistance was found between picoxystrobin and fungicides with different modes of action. In addition, the fitness of picoxystrobin-resistant strains significantly decreased as compared to picoxystrobin-sensitive strains, suggesting that the resistance risk of *N. clavispora* to picoxystrobin is medium to high. Taken together, our data provide critical information for the application of chemical fungicides in vine tea leaf blight management.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13051340/s1. Figure S1: Effect of 0.56 μg/mL picoxystrobin on cell membrane permeability of three wild-type N. clavispora strains (ZJJ5, ZJJ9, and ZJJ12). Bars denote the stand error of two experiments; Figure S2: Sequence analysis of Cytb in picoxystrobin-sensitive strain ZJJ5 and picoxystrobin-resistant strains ZJJ5-K2, ZJJ5-K3, ZJJ5-K5; Table S1: Concentrations of differnt fungicides; Table S2: Sequence obtained from GenBank of the Neopestalotiopsis isolates that used in the phylogenetic study; Table S3: Resistance factor and resistance stability of picoxystrobin-resistant mutants of N. clavispora.

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References

1. Yang, C.S.; Chen, G.; Wu, Q. Recent scientific studies of a traditional Chinese medicine, tea, on prevention of chronic diseases. *J. Tradit. Complement. Med.* **2014**, *4*, 17–23. [CrossRef] [PubMed]
2. Patel, S.; Sharma, V.; Chauhan, N.S.; Thakur, M.; Dixit, V.K. Hair growth: Focus on herbal therapeutic agent. *Curr. Drug Discov. Technol.* **2015**, *12*, 21–42. [CrossRef] [PubMed]
3. Zhang, Q.L.; Zhao, Y.F.; Zhang, M.Y.; Zhang, Y.L.; Ji, H.F.; Shen, L. Recent advances in research on vine tea, a potential and functional herbal tea with dihydromyricetin and myricetin as major bioactive compounds. *J. Pharm. Anal.* **2021**, *11*, 555–563. [CrossRef] [PubMed]
4. Wang, C.; Xiong, W.; Perumalla, S.R.; Fang, J.G.; Sun, C.C. Solid-state characterization of optically pure (+) dihydromyricetin extracted from *Ampelopsis grossedentata* leaves. *Int. J. Pharm.* **2016**, *511*, 245–252. [CrossRef] [PubMed]
5. Xie, K.; He, X.; Chen, K.Y.; Chen, J.H.; Sakao, K.; Hou, D. Antioxidant properties of a traditional vine tea, *Ampelopsis grossedentata*. *Antioxidants* **2019**, *8*, 295. [CrossRef] [PubMed]

6. Carneiro, R.C.V.; Wang, H.J.; Duncan, S.E.; O'Keefe, S.F. Flavor compounds in vine tea (*Ampelopsis grossedentata*) infusions. *Food Sci. Nutr.* **2020**, *8*, 4505–4511. [CrossRef] [PubMed]
7. Ye, L.; Wang, H.; Duncan, S.E.; Eigel, W.N.; O'Keefe, S.F. Antioxidant activities of vine tea (*Ampelopsis grossedentata*) extract and its major component dihydromyricetin in soybean oil and cooked ground beef. *Food Chem.* **2015**, *172*, 416–422. [CrossRef]
8. Zhang, X.; Xu, Y.; Xue, H.; Jiang, G.C.; Liu, X.J. Antioxidant activity of vine tea (*Ampelopsis grossedentata*) extract on lipid and protein oxidation in cooked mixed pork patties during refrigerated storage. *Food Sci. Nutr.* **2019**, *7*, 1735–1745. [CrossRef]
9. Bartlett, D.W.; Clough, J.M.; Godwin, J.R.; Hall, A.A.; Hamer, M.; Parr-Dobrzanski, B. The strobilurin fungicides. *Pest Manag. Sci.* **2002**, *58*, 649–662. [CrossRef]
10. Sabbahi, R.; Oliveira, D.; Marceau, J. Influence of honey bee (Hymenopetra: Apidae) density on the production of canola (Cruciferae: Brassicaceae). *J. Econ. Entomol.* **2005**, *98*, 367–372. [CrossRef]
11. Keith, L.M.; Velasquez, M.E.; Zee, F.T. Identification and characterization of *Pestalotiopsis* spp. causing scab disease of guava, *Psidium guajava*, in Hawaii. *Plant Dis.* **2006**, *90*, 16–23. [CrossRef] [PubMed]
12. Chen, Y.; Zeng, L.; Shu, N.; Jiang, M.; Wang, H.; Huang, Y.; Tong, H. *Pestalotiopsis*-like species causing gray blight disease on *Camellia sinensis* in China. *Plant Dis.* **2018**, *102*, 98–106. [CrossRef] [PubMed]
13. Shu, J.; Yu, Z.H.; Sun, W.X.; Zhao, J.; Li, Q.L.; Tang, L.H.; Guo, T.X.; Huang, S.P.; Mo, J.Y.; Hsiang, T.; et al. Identification and characterization of *Pestalotioid* fungi causing leaf spots on mango in southern China. *Plant Dis.* **2020**, *104*, 1207–1213. [CrossRef] [PubMed]
14. Udayanga, D.; Castlebury, L.A.; Rossman, A.Y.; Chukeatirote, E.; Hyde, K.D. Insights into the genus *Diaporthe*: Phylogenetic species delimitation in the *D. eres* species complex. *Fungal Divers.* **2014**, *67*, 203–229. [CrossRef]
15. Duan, Y.B.; Ge, C.Y.; Liu, S.M.; Chen, C.J.; Zhou, M.G. Effect of phenylpyrrole fungicide fludioxonil on morphological and physiological characteristics of *Sclerotinia sclerotiorum*. *Pestic. Biochem. Physiol.* **2013**, *106*, 61–67. [CrossRef]
16. Duan, Y.B.; Ge, C.Y.; Liu, S.M.; Wang, J.X.; Zhou, M.G. A two-component histidine kinase Shk1 controls stress response, sclerotial formation and fungicide resistance in *Sclerotinia sclerotiorum*. *Mol. Plant Pathol.* **2013**, *14*, 708–718. [CrossRef]
17. Duan, Y.B.; Xiu, Q.; Li, H.R.; Li, T.; Wang, J.X.; Zhou, M.G. Pharmacological characteristics and control efficacy of a novel SDHI fungicide pydiflumetofen against *Sclerotinia sclerotiorum*. *Plant Dis.* **2019**, *103*, 77–82. [CrossRef]
18. Li, T.; Xiu, Q.; Zhang, J.; Wang, J.X.; Duan, Y.B.; Zhou, M.G. Pharmacological characteristics and efficacy of fluazinam against *Corynespora cassiicola*, causing cucumber target spot in greenhouses. *Plant Dis.* **2020**, *104*, 2449–2454. [CrossRef]
19. Yuan, S.Q.; Wang, Y.C.; Lei, L.; Hong, J.Y.; Yi, T.Y.; Hong, Y.Y. First report of *Pestalotiopsis microspora* causing leaf spot on moyeam in China. *Plant Dis.* **2021**, *106*, 1996. [CrossRef]
20. Bi, Y.; Cui, X.L.; Lu, X.L.; Cai, M.; Liu, X.L.; Hao, J.J.J. Baseline sensitivity of natural population and resistance of mutants in Phytophthora capsici to zoxamide. *Phytopathology* **2011**, *101*, 1104–1111. [CrossRef]
21. Mao, X.W.; Wu, Z.W.; Bi, C.W.; Wang, J.X.; Zhao, F.F.; Gao, J.; Hou, Y.P.; Zhou, M.G. Molecular and biochemical characterization of pydiflumetofen resistant isolates of *Didymella bryoniae*. *J. Agric. Food Chem.* **2020**, *68*, 9120–9130. [CrossRef] [PubMed]
22. Brandt, U.; Schagger, H.; Jagow, G. Characterisation of binding of the methox-yacrylate inhibitors mitochondrial cytochrome c reductase. *Eur. J. Biochem.* **1988**, *173*, 499–506. [CrossRef] [PubMed]
23. Qu, X.P.; Li, J.S.; Wang, J.X.; Wu, L.Y.; Wang, Y.F.; Chen, C.J.; Zhou, M.G.; Hou, Y.P. Effects of the dinitroaniline fungicide fluazinam on *Fusarium fujikuroi* and rice. *Pestic. Biochem. Physiol.* **2018**, *152*, 98–105. [CrossRef] [PubMed]
24. Jones, P.J.; Jew, S. Functional food development: Concept to reality. *Trends Food Sci. Technol.* **2007**, *18*, 387–390. [CrossRef]
25. Gao, J.; Liu, B.; Ning, Z.; Zhao, R.; Zhang, A.; Wu, Q. Characterization and antioxidant activity of flavonoid-rich extracts from leaves of *Ampelopsis grossedentata*. *J. Food Biochem.* **2009**, *33*, 808–820. [CrossRef]
26. Zheng, X.J.; Xiao, H.; Zeng, Z.; Sun, Z.W.; Lei, C.; Dong, J.Z.; Wang, Y. Composition and serum antioxidation of the main flavonoids from fermented vine tea (*Ampelopsis grossedentata*). *J. Funct. Foods* **2014**, *9*, 290–294. [CrossRef]
27. Xie, J.; Wei, J.G.; Wang, K.W.; Luo, J.; Wu, Y.J.; Luo, J.T.; Yang, X.H.; Yang, X.B. Three phytotoxins produced by *Neopestalotiopsis clavispora*, the causal agent of ring spot on *Kadsura coccinea*. *Microbiol. Res.* **2020**, *238*, 126531. [CrossRef]
28. Shi, J.; Zhang, X.; Liu, Y.; Zhang, Z.; Wang, Z.; Xue, C.; Ma, Y.; Wang, F. First report of *Neopestalotiopsis clavispora* causing calyx and receptacle blight on strawberry in China. *Plant Dis.* **2022**, *106*, 1307. [CrossRef]
29. Cowger, C.; Meyers, E.; Whetten, R. Sensitivity of the U.S. wheat powdery mildew population to quinone outside inhibitor fungicides and determination of the complete *Blumeria graminis* f. sp. *Tritici* cytochrome b gene. *Phytopathology* **2022**, *112*, 249–260. [CrossRef]
30. Abate, M.; Festa, A.; Falco, M.; Lombardi, A.; Luce, A.; Grimaldi, A.; Zappavigna, S.; Sperlongano, P.; Irace, C.; Caraglia, M.; et al. Mitochondria as playmakers of apoptosis, autophagy and senescence. *Semin. Cell Dev. Biol.* **2020**, *98*, 139–153. [CrossRef]
31. van der Bliek, A.M.; Sedensky, M.M.; Morgan, P.G. Cell biology of the mitochondrion. *Genetics* **2017**, *207*, 843–871. [CrossRef] [PubMed]
32. Duan, Y.B.; Lu, F.; Zhou, Z.H.; Zhao, H.H.; Zhang, J.; Mao, Y.S.; Li, M.X.; Wang, J.X.; Zhou, M.G. Quinone outside inhibitors affect DON biosynthesis, mitochondrial structure and toxisome formation in *Fusarium graminearum*. *J. Hazard. Mater.* **2020**, *398*, 122908. [CrossRef] [PubMed]
33. Chen, Y.L.; Mao, X.W.; Wang, J.X.; Wu, L.Y.; Zhou, M.G.; Hou, Y.P. Activity of the dinitroaniline fungicide fluazinam against *Bipolaris maydis*. *Pestic. Biochem. Physiol.* **2018**, *148*, 8–15. [CrossRef] [PubMed]
34. Mao, X.W.; Wang, Y.F.; Hou, Y.P.; Zhou, M.G. Activity of the succinate dehydrogenase inhibitor fungicide penthiopyrad against *Sclerotinia sclerotiorum*. *Plant Dis.* **2020**, *104*, 2696–2703. [CrossRef] [PubMed]
35. Chen, C.J.; Yu, J.J.; Bi, C.W.; Zhang, Y.N.; Xu, J.Q.; Wang, J.X.; Zhou, M.G. Mutations in a beta-tubulin confer resistance of *Gibberella zeae* to benzimidazole fungicides. *Phytopathology* **2009**, *99*, 1403–1411. [CrossRef]

36. Sun, H.Y.; Wang, H.C.; Chen, Y.; Li, H.X.; Chen, C.J.; Zhou, M.G. Multiple resistance of *Botrytis cinerea* from vegetable crops to carbendazim, diethofencarb, procymidone, and pyrimethanil in China. *Plant Dis.* **2010**, *94*, 551–556. [CrossRef]
37. Duan, Y.B.; Yang, Y.; Wang, J.X.; Chen, C.J.; Steinberg, G.; Fraaije, B.A.; Zhou, M.G. Simultaneous detection of multiple benzimidazole-eesistant β-tubulin variants of *Botrytis cinerea* using loop-mediated isothermal amplification. *Plant Dis.* **2018**, *102*, 2016–2024. [CrossRef]
38. Hou, Y.P.; Qu, X.P.; Mao, X.W.; Kuang, J.; Duan, Y.B.; Song, X.S.; Wang, J.X.; Chen, C.J.; Zhou, M.G. Resistance mechanism of *Fusarium fujikuroi* to phenamacril in the field. *Pest Manag. Sci.* **2018**, *74*, 607–616. [CrossRef]
39. Yang, Y.; Li, M.X.; Duan, Y.B.; Li, T.; Shi, Y.Y.; Zhao, D.L.; Zhou, Z.H.; Xin, W.J.; Wu, J.; Pan, X.Y.; et al. A new point mutation in β_2-tubulin confers resistance to carbendazim in *Fusarium asiaticum*. *Pestic. Biochem. Physiol.* **2018**, *145*, 15–21. [CrossRef]
40. Degli Esposti, M.; De Vries, S.; Crimi, M.; Ghelli, A.; Patarnello, T.; Meyer, A. Mitochondrial cytochrome b: Evolution and structure of the protein. *Biochim. Biophys. Acta* **1993**, *1143*, 243–271. [CrossRef]
41. Gisi, U.; Sierotzki, H.; Cook, A.; McCaffery, A. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Manag. Sci.* **2002**, *58*, 859–867. [CrossRef] [PubMed]
42. Fernandez-Ortuno, D.; Tores, J.A.; de Vicente, A.; Perez-Garcia, A. Field resistance to QoI fungicides in *Podosphaera fusca* is not supported by typical mutations in the mitochondrial cytochrome b gene. *Pest Manag Sci.* **2008**, *64*, 694–702. [CrossRef] [PubMed]
43. Fernandez-Ortuno, D.; Tores, J.A.; de Vicente, A.; Perez-Garcia, A. Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. *Int. Microbiol.* **2008**, *11*, 1–9. [PubMed]
44. Qiu, J.B.; Huang, T.T.; Xu, J.Q.; Bi, C.W.; Chen, C.J.; Zhou, M.G. β-tubulins in *Gibberella zeae*: Their characterization and contribution to carbendazim resistance. *Pest Manag. Sci.* **2012**, *68*, 1191–1198. [CrossRef]
45. Zhang, L.G.; Li, B.C.; Zhang, Y.; Jia, X.J.; Zhou, M.G. Hexokinase plays a critical role in deoxynivalenol (DON) production and fungal development in *Fusarium graminearum*. *Mol. Plant Pathol.* **2016**, *17*, 16–28. [CrossRef]
46. Zhou, Z.H.; Duan, Y.B.; Zhou, M.G. Carbendazim-resistance associated β_2-tubulin substitutions increase deoxynivalenol biosynthesis by reducing the interaction between β_2-tubulin and IDH3 in *Fusarium graminearum*. *Environ. Microbiol.* **2020**, *22*, 598–614. [CrossRef]
47. Duan, Y.B.; Yang, Y.; Wang, J.X.; Liu, C.C.; He, L.L.; Zhou, M.G. Development and application of loop-mediated isothermal amplification for detecting the highly benzimidazole-resistant isolates in *Sclerotinia sclerotiorum*. *Sci. Rep.* **2015**, *5*, 17278. [CrossRef]
48. Shao, W.Y.; Zhao, Y.F.; Ma, Z.H. Advances in understanding fungicide resistance in *Botrytis cinerea* in China. *Phytopathology* **2021**, *111*, 455–643. [CrossRef]
49. Chin, K.M.; Chavaillaz, D.; Kaesbohrer, M.; Staub, T.; Felsenstein, F.G. Characterizing resistance risk of *Erysiphe graminis* f.sp. *tritici* to strobilurins. *Crop Protect.* **2001**, *20*, 87–96. [CrossRef]