STABILITY AND ACTION MECHANISM OF BIOACTIVE COMPOUND CYCLOHEXIMIDE FROM *STREPTOMYCES ATRATUS* PY-1 AGAINST *PLASMOPARA VITICOLA* TO CONTROL GRAPEVINE DOWNY MILDEW

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(Received 20th Aug 2024; accepted 9th Dec 2024)

Abstract. Downy mildew (*Plasmopara viticola*) is one of the most serious oomycete diseases. This disease can be found in all grape-growing countries. The metabolites of *Streptomyces atratus* PY-1 were confirmed to contain bioactive cycloheximide, which strongly inhibits *P. viticola.* In this study, the bioactive cycloheximide compound was extracted from the fermentation filtrate of PY-1 by dichloromethane extraction, column chromatography, and semi-preparative high-performance liquid chromatography and confirmed that the cycloheximide bioactive compound inhibited *P. viticola in vitro* by 90.06%, 81.74%, and 62.41% with concentrations of 10^4, 10^2 and 10 μg/mL, respectively. The active substances caused the sporangia and sporangiophores of *P. viticola* to fold, rupture and lose their ability to infect. They had a high degree of physical and chemical stability. The activity of the bioactive compounds was relatively stable when stored in an environment of pH < 4 and pH > 8 below 70 °C, directly irradiated by a UV lamp (15 W) for < 84 h, and stored in the dark for 6 months at room temperature. In the field, the control effect of 10 mg/mL of the cycloheximide bioactive compound against grapevine downy mildew was slightly less than that of a 2,000-fold diluent of 52.5% oxazolidone and hydrocyanide water dispersible granule but significantly higher than that of a 1,000-fold diluent of 58% metalaxyl and mancozeb wettable powder. Therefore, *S. atratus* could be used as a potential biocontrol agent to control grapevine downy mildew.
Keywords: *biological control, antibiotics, Plasmopara viticola, inhibition mechanism, control efficiency*

Introduction

Grapevine downy mildew caused by *Plasmopara viticola* is one of the most serious diseases in grape production all over the world (Gessler et al., 2011; Zhang et al., 2020), and it is also one of the top 10 oomycete diseases in the world (Kamoun et al., 2015). Currently, chemical fungicides are the most effective method for controlling grapevine downy mildew. Among many chemical agents, copper agents, such as the Bordeaux mixture, are the most effective. The copper agents have been used in vineyards for more than 150 years, and the dosage is 80 kg/ha per year (Rusjan et al., 2007). Copper chemicals have had a broad-spectrum control effect for a long time on grapevine downy mildew and other plant pathogenic fungi and bacterial diseases, which made it hard to replace them in field disease control. Currently, in most orchards, copper agents are typically sprayed once a week to effectively control downy mildew (Caffi et al., 2016). However, copper agents are difficult to transfer and degrade. The extensive use of copper agents gradually increases the concentration of copper in the soil. As a result, they could have increasingly significant toxic effects and cause residue problems (Flemming and Trevors, 1989; Komárek et al., 2010). Synthetic fungicides are detrimental to the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2301_15771587

environment, and the application of current biocontrol agents (BCAs) have resulted in disease resistance (Ahsan et al., 2022). Today, biological disease control is recognized by the scientific community as an important tool of integrated pest management (IPM). However, biocontrol is hindered by the absence of efficient, commercially available BCAs. A critical step in developing commercial biocontrol products is the identification of new BCAs, which requires rapid and robust screening methods that can quickly evaluate large number of BCA candidates (Raymaekers et al., 2020).

Bioactive compounds produced by *S. atratus* PY-1 have strong inhibitory activity against *P. viticola* (Liang et al., 2016; Zang et al., 2018). In this study, cycloheximide bioactive compounds were purified from the fermentation filtrate of *S. atratus* PY-1. The cycloheximide compound was subjected to stability tests, such as temperature, light, storage time and in-field experiments, and bioassayed against *P. viticola*. The results would provide data support for the development of a biological control agent of grapevine downy mildew.

Materials and Methods

Extraction and antimicrobial activity of the S. atratus PY-1 cycloheximide bioactive compound

S. atratus PY-1 was streaked on potato dextrose agar (PDA) media and cultured at 28°C for 5 days. It was then inoculated into a 250 mL Erlenmeyer flask that contained 100 mL of fermentation medium and cultured at 28°C for 3 days and shaken with 180 rpm. The fermentation broth of strain PY-1 was obtained by inoculating 5% of the seed liquid into a 250 mL flask with 90 mL potato dextrose broth (PDB), which was shaken at 180 rpm for 5 days at 28°C. The fermentation filtrate of PY-1 was centrifuged at 4°C and 10000 rpm for 15 min, and the supernatant was filtered through a 0.45 μm microporous membrane.

The fermentation filtrate was extracted by dichloromethane with a separatory funnel. The organic phase was concentrated by rotary evaporation at 37°C. The concentrate was separated and purified by silica gel column chromatography and Sephadex LH-20 column chromatography. The components with strong activity were further separated by Agilent 1100 high-performance liquid chromatography (HPLC) (Agilent Technologies, Santa Clara, CA, USA) under the following conditions: Mobile phase: 37-48% CH_3OH-H_2O, injection volume: 10 μL, flow rate: 2 mL/min, time: 0-40 min, detection wavelength: 210 nm, column temperature: room temperature, components with a retention time of 19-20.5 min were collected, and concentrated by rotary evaporation (Liang et al., 2016).

Bioassay against P. viticola

The active substances were dissolved with dimethyl sulfoxide (DMSO) and diluted to 10^4, 10^2 and 10 μg/mL with sterile water. Grape leaves of the same leaf age and size were selected, and the petioles were wrapped with moistened cotton. The back of the leaf was upward and placed in a petri dish with wet filter paper. A 1 mL suspension of *P. viticola* with a concentration of 10^5 sporangia per mL was evenly sprayed on the back of a leaf and sealed with parafilm. The leaves were placed in an incubator at 22°C and cultured at 12 h/12 h light/dark. After 24 h, different concentrations of the compound were equally sprayed on each leaf in a volume of 1 mL. Ten leaves each were treated, and the experiment was conducted in triplicate. Sterile water with the same amount of DMSO

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was sprayed as the control. The incidence of disease was observed, and the control effect was calculated after 7 days. The control effect was calculated using a disease index. The disease severity was assessed using a 6-point scale based on the area of the leaves covered in white lesions: 0 (no symptoms); 1 (below 5%); 3 (6 to 25%); 5 (26 to 50%); 7 (51 to 75%); and 9 (more than 75%) (Yu et al., 2016).

The control effect was calculated according to the following formula (Zang et al., 2018):

$$\mathrm{DI} = \frac{\sum(A \times B)}{M \times Bmax} \times 100 \quad \text{(Eq.1)}$$

where DI- disease index; *A*-the number of diseased leaves from all the levels; *B*-the level of each diseased leaf; *M*-the total number of the leaves, and *Bmax*-the highest level of the disease.

$$\mathrm{I}(\%) = \frac{Zck \times \mathrm{Z}x}{Zck} \times 100 \quad \text{(Eq.2)}$$

where I-the control effect; *Zck*-the disease index of control group, and *Zx*-the disease index of the treatment group.

Stability analysis of the PY-1 cycloheximide bioactive compound

The effects of temperature, pH, ultraviolet (UV) rays and storage time on the stability of the antimicrobial active substance were determined. (1) A solution of 1 mg/mL was treated at 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100℃ for 30 min in a water bath and autoclaved at 121℃ for 15 min; (2) The pH of cycloheximide bioactive compound solution (1 mg/mL) was adjusted to pH 3, 4, 5, 6, 7, 8, 9 and 10 with 0.1 mol/L of HCl or NaOH and treated for 30 min; (3) An UV lamp with power of 15 W was used to irradiate a 1 mg/mL solution of the bioactive compound at a height of 20 cm for 12, 24, 36, 48, 60, 72, 84 and 96 h; and (4) The compound was stored at room temperature in the dark for 1, 2, 3, 4, 5 and 6 months. The antimicrobial activity of these different treatments was tested against *P. viticola* to analyze the stability of active substances.

Effect of the PY-1 cycloheximide bioactive compound on P. viticola sporangia

A sporangial suspension of *P. viticola* was inoculated on the back of grape leaves as described in Section 2.1. When the frosty mildew layer appeared on the back of the leaves, 20 μL of a 1 mg/mL solution of the PY-1 cycloheximide bioactive compound was inoculated on the downy mildew layer. After 0, 2, 6, 12 and 24 h, 4 mm×4 mm lobules were cut at the edges of the inoculation point with a blade, fixed with 2% glutaraldehyde for 4 h, washed three times with normal saline, dehydrated by an ethanol gradient, and then the dried and sticky sample was coated by an ion sputtering apparatus. A sterile aqueous solution that contained the same amount of DMSO was added as the control treatment. The effect of cycloheximide bioactive compound on *P. viticola* was observed by a Hitachi S4800 scanning electron microscope (SEM) (Tokyo, Japan).

Determination of the field control effect of the PY-1 cycloheximide bioactive compound

The experiment was conducted in the same field with serious grapevine downy mildew all year round. This experiment was conducted for two consecutive years from 2020 to

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2021, and it was designed according to the field test criteria stipulated by the Institute of Drug Control of the Ministry of Agriculture, Beijing, China, with a total of six treatments: Cycloheximide bioactive compound with concentrations of 10, 1 and 0.1 mg/mL were treated as three treatments; the fungicides 52.5% oxazolidone and hydrocyanide water dispersible granule (produced by Jiangxi Zhonghe Chemical Co., Ltd., Nanjing, China) and 58% metalaxyl and mancozeb wettable powder (produced by Sichuan Runer Technology Co., Ltd., CITY, Australia) were used as the chemical agent control. The blank control was clear water (*Table 1*). When the scabs of grapevine downy mildew disease first appeared in the field, all the dilutions were sprayed evenly. Each treatment was 10 grape plants repeated four times. There were 24 test plots in a random order (*Figure 1*). Ten days after the last application, the incidence stages of grapevine downy mildew of all leaves on 10 new vines were randomly investigated in each experimental site, and the number of diseased leaves at all levels was recorded. The disease index was used to calculate the control effect as described in Section *2.1*.

Table 1. *The control effect of cycloheximide bioactive compound with different concentrations against the grapevine downy mildew in the field*

Letter	Treatment
A1	Active substance of PY-1 with 10 mg/mL
A2	Active substance of PY-1 with 1 mg/mL
A3	Active substance of PY-1 with 0.1 mg/mL
B	52.5% Oxazolidone and hydrocyanide WG diluted 2000 times
C	58% Metalaxyl and mancozeb WP diluted 1000 times
D	Water

Note: A1, A2, A3 were each treatment of biocontrol agents PY-1. B and C were CK of chemical agents. D was blank control

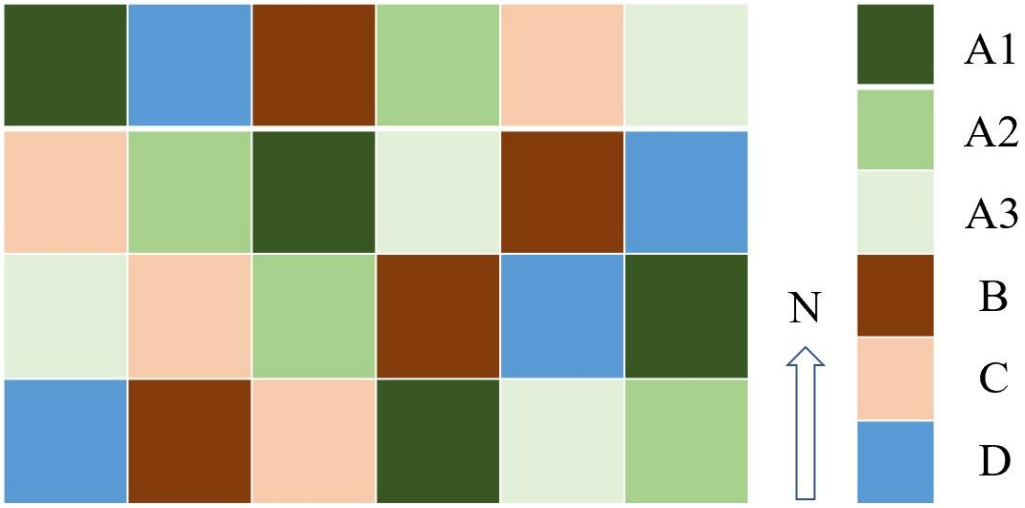

Figure 1. *The layout of biological control efficiency of cycloheximide bioactive compound against grapevine downy mildew in the field assay. A1, A2, A3 were cycloheximide bioactive compound with concentration of 10 mg/mL, 1 mg/mL and 0.1 mg/mL respectively; B was 52.5% Oxazolidone and hydrocyanide WG diluted 2000 times; C was 58% Metalaxyl and mancozeb WP diluted 1000 times; D was the control of clear water*

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Statistical analysis

The test data were statistically analyzed by Microsoft Excel 2016 (Redmond, WA, USA) and SPSS 24.0 (IBM, Inc., Armonk, NY, USA). Critical difference and Duncan's multiple range tests were utilized to compare the means. A difference between two means at $P < 0.05$ was considered significantly different.

Results

Extraction and antimicrobial activity of the cycloheximide bioactive compound in the fermentation filtrate of PY-1

The cycloheximide bioactive compound was extracted from the fermentation filtrate of *S. atratus* PY-1 by extraction, column chromatography and Agilent 1100 HPLC, and 1.03 g was purified. The cycloheximide bioactive compound strongly inhibited *P. viticola* (*Figure 2*). The control effects with concentrations of 10^4, 10^2 and 10 μg/mL against downy mildew were 90.06%, 81.74% and 62.41% in vitro, respectively (*Figure 3*).

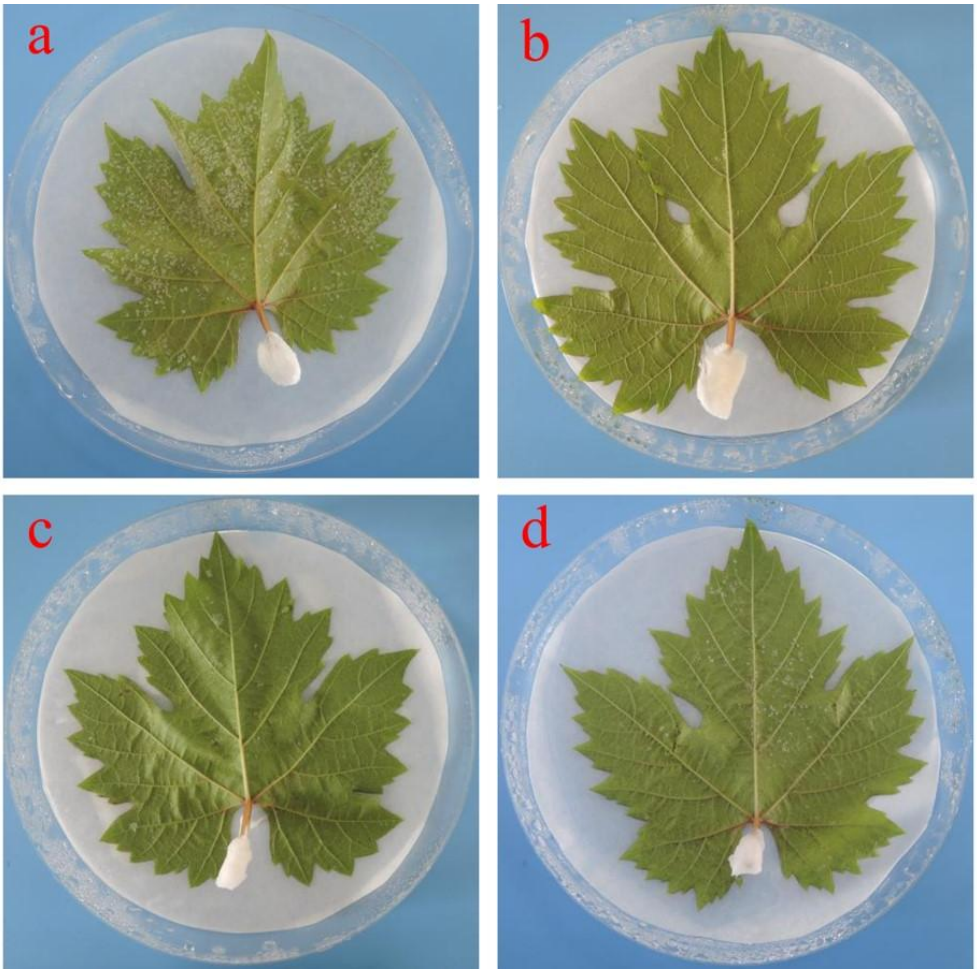

Figure 2. *Inhibitory activity of cycloheximide bioactive compound with different concentrations against P. viticola in vitro. Emage a was control of clear water; b, c, d were inhibitory activity of cycloheximide bioactive compound with the concentration of 10^4, 10^2 and 10 μg/mL against P. viticola*

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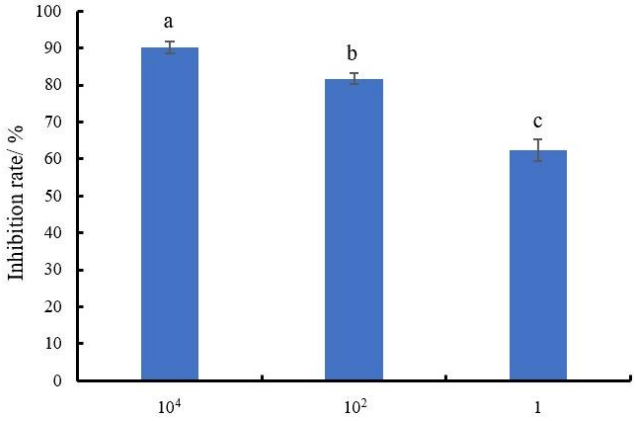

Figure 3. *The inhibition rate of cycloheximide bioactive compound with the concentration of 10^4, 10^2 and 10 μg/mL against grape grown mildew were 90.06%, 81.74% and 62.41% in vitro, respectively*

Stability analysis of the cycloheximide bioactive compound

The stability of cycloheximide bioactive compound was analyzed by testing the effects of different temperatures, pH, UV and storage time on the antimicrobial activity against *P. viticola*. The results showed that the bioactive compound was stable when stored at < 70°C (*Figure 4a*). It was insensitive to acidic environments and sensitive to alkaline environments. Its antimicrobial activity decreased significantly when it was placed at pH>8 (*Figure 4b*). The activity was stable when the direct irradiation time of the bioactive compound from a 15W UV lamp < 84 h (*Figure 4c*). The antimicrobial activity of the bioactive substance that had been stored in the dark at room temperature for 6 months remained the same (*Figure 4d*). These results showed that the cycloheximide bioactive compound from the fermentation filtrate of *S. atratus* PY-1 was stable at different environments, and strain PY-1 could be used as a potential biocontrol factor to control grapevine downy mildew.

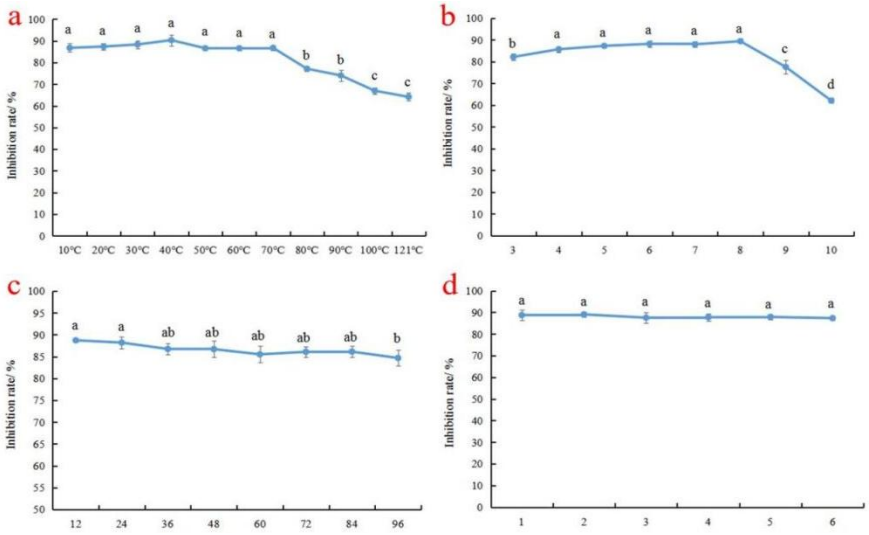

Figure 4. *Stability analysis of cycloheximide bioactive compound. a, b, c, d meant effect of temperature, pH, ultraviolet ray and store time on cycloheximide bioactive compound respectively*

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DOI: http://dx.doi.org/10.15666/aeer/2301_15771587

Effect of the PY-1 cycloheximide bioactive compound on P. viticola sporangia

The observation results of SEM showed that the untreated sporangia and sporangiophores of *P. viticola* were full and smooth. After treatment with bioactive compound (1 mg/mL) for more than 2 h, the sporangia and sporangiophores of *P. viticola* appeared constricted, ruptured and deformed to varying degrees (*Figure 5*). This showed that the bioactive compound could directly affect the sporangia and sporangiophores of *P. viticola*. It made them lose their infectivity and prevented the continuous expansion of disease spots in the diseased tissues.

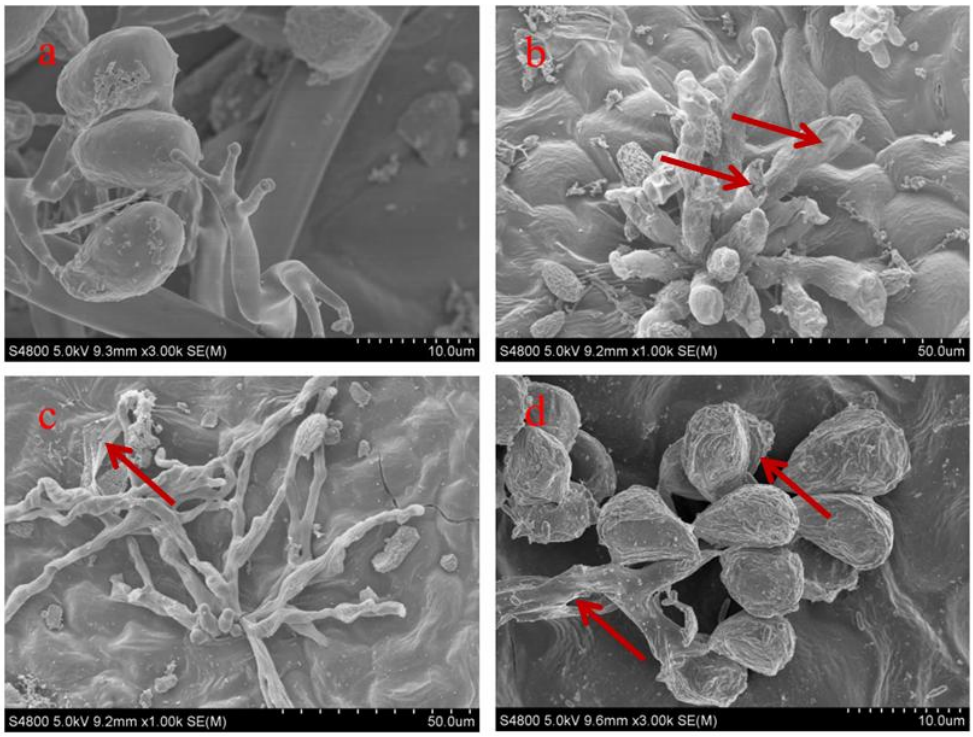

Figure 5. *Scanning electron micrographs (SEM) demonstrating the effect of cycloheximide bioactive compound purified from fermentation liquor of strain PY-1 on sporangia and sporangiophore of P. viticola. Emage a was the sporangium and sporangiophore of P. viticola untreated with cycloheximide bioactive compound were full and smooth; and b, c, d were the sporangia and sporangiophore of P. viticola appeared constriction, rupture and deformity in varying degrees after being treated with bioactive compound (1 mg/mL) for more than 2 hours. The arrow marked the constriction, rupture and deformity of sporangia and sporangiophores of P. viticola*

Field control effect of the PY-1 cycloheximide bioactive compound against grapevine downy mildew

The cycloheximide bioactive compound of *S. atratus* PY-1 showed the desired control effect against grapevine downy mildew in fields in 2020 and 2021. The cycloheximide bioactive compound controlled downy mildew at a concentration of 10 mg/mL on grapevine downy mildew by 82.34% and 86.01% in 2020 and 2021, respectively. The control effects of 52.5% oxazolidone and hydrocyanide water dispersible granule diluted 2,000-fold were 87.26% and 86.99%, respectively. In 2021, the field control effect of 10 mg/mL bioactive compound was not significantly different from that of 52.5%

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2301_15771587

oxazolidone and hydrocyanide water dispersible granule diluted 2,000-fold. However, it was significantly higher than that of 58% metalaxyl and mancozeb wettable powder diluted 1,000-fold (71.73% and 66.67%, respectively). The control effect of 1 mg/mL bioactive compound on grapevine downy mildew in 2020 and 2021 was not significantly different from that of 58% metalaxyl and mancozeb wettable powder diluted 1,000-fold (*Table 2*).

Table 2. *The control effect of cycloheximide bioactive compound with different concentrations against the grapevine downy mildew in the field*

Treatment	2020		2021	
	Disease index	Control effect（%）	Disease index	Control effect（%）
A1	2.69	82.34±2.66 b	2.13	86.01±2.21 ab
A1	4.44	70.88±2.94 cd	4.74	68.90±2.82 cd
A1	6.32	58.55±1.73 e	5.90	61.31±2.27 e
B	1.94	87.26±2.98 a	1.98	86.99±1.60 a
C	4.31	71.73±2.08 c	5.08	66.67±3.76 d
D	15.24		10.33	

The means followed by the different letters-significantly different (P<0.05) according to Critical difference and Duncan's multiple range tests

Conclusions

Cycloheximide bioactive compound, extracted from the fermentation filtrate of *S. atratus* PY-1 would cause the sporangia and sporangiophores of *P. viticola* to fold, rupture and lose their ability to infect. And it showed strong inhibitor activity against *P. viticola*, and confirmed that the it inhibited *P. viticola in vitro* by 90.06%, 81.74%, and 62.41% with concentrations of 10^4, 10^2 and 10 μg/mL, respectively. Excitedly, it showed excellent control efficiency against grapevine downy mildew in two years' field experiments. The activity of cycloheximide bioactive compound was relatively stable when stored in condition of pH < 4 and pH > 8 below 70 ℃, directly irradiated by a UV lamp (15 W) for < 84 h, and stored in the dark for 6 months at room temperature. Therefore, both *S. atratus* PY-1 and its metabolites have the potential to be developed into biocontrol agents against grapevine downy mildew.

Discussion

In this study, the cycloheximide bioactive compound was purified from the fermentation filtrate of *S. atratus* PY-1 by solvent extraction, column chromatography and semi-preparative liquid chromatography. The antimicrobial activity against *P. viticola* was though an in vitro leaf method, and the stability of the crude extract of the active substance was analyzed. The results showed that the cycloheximide bioactive compound strongly and stably inhibited *P. viticola*.

Cycloheximide, as a broad-spectrum antibiotic, can inhibit most eukaryotes *in vitro*. Now, it is primarily used to control plant diseases and pests (Pan et al., 2019; Nakae et al., 2000). It had been reported that 10% of cycloheximide diluted 150-250-fold was used to effectively control *Colletotrichum camelliae* (Shen, 1981). Cycloheximide, isolated from the metabolites of *S. yunnanensis* YIM41004T, strongly inhibited the spore germination and mycelial growth of *Phytophthora parasitica* var. *nicotianae*, and the

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control effect of tobacco black shank disease was 75% (Xia et al., 2007). In addition, cycloheximide also had anti-bacterial, anti-viral, anti-protozoan, anti-tumor and herbicidal effects (Sonoda et al., 1991; Sugawara et al., 1992; Hoagland, 2001; Li et al., 2001; Lim et al., 2009; Wrona et al., 2010). In the field of medicine and molecular biology, it is used to inhibit protein synthesis and is commonly used in research on protein expression, cell cycle metabolic regulation, apoptosis mechanism and pathological model design (Doyle et al., 2010; Fischer-Posovszky et al., 2011; Poutrain et al., 2011).

Cycloheximide was first identified in the fermentation product of *Streptomyces griseus* in the 1940s (Leach, 1947). In this study, the cycloheximide bioactive compound in the fermentation filtrate of *S. atratus* PY-1 can destroy the sporangia and sporangiophores of *P. viticola*, resulting in pathogen death and the loss of its ability to cause infection. The mechanism of cycloheximide has been shown to be the inhibition of protein synthesis by eukaryotes by its specific binding to the 60S subunit of eukaryote ribosome. This terminates the extension of protein translation chain (Siegel and Sisler, 1963; Ennis, 1968; Ju et al., 2005; Huang et al., 2011).

The cycloheximide bioactive compound produced by *S. atratus* PY-1 causes *P. viticola* to lose its infectivity by destroying the sporangia and sporangiophores, which successfully controls grapevine downy mildew. Simultaneously, the active substance effectively controls grapevine downy mildew in the field. Therefore, *S. atratus* PY-1 could be used as a potential biocontrol agent for the control of grapevine downy mildew, which provides a new way for the biological control of grapevine downy mildew.

Acknowledgments. This research was funded by National Key Research and Development Program of China (2017YFD0201104), Grape Science and Technology Special Mission of Beizhen City of Liaoning Province (2024JH5/10400074).

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