APPLICATION OF *BACILLUS SUBTILIS* AND TEBUCONAZOLE COMPOUND IN PREVENTING AND TREATING FRAGRANT PEAR VALSA CANKER

Field of the Invention

[01] The present invention relates to the technical field of pest and disease control, and in particular to an application of a *Bacillus subtilis* and tebuconazole compound in preventing and treating Fragrant Pear Valsa Canker.

Background to the Invention

[02] Fragrant Pear Valsa Canker (also known as bark rot or foul-smelling bark disease) is a devastating fungal disease caused by pathogens such as *Cytospora pyri*, *C. nivea*, and *C. chrysosperma*. It mainly affects the trunk, primary branches, and lateral branches of pear trees, leading to bark decay and decline in tree vigor, thereby severely reducing the yield and quality of fragrant pears. The disease is particularly rampant in the Korla producing area of Xinjiang, posing a significant threat to the local pillar fruit industry.

[03] At present, measures for preventing and treating the disease mainly rely on chemical fungicides, such as tebuconazole. Tebuconazole disrupts the fungal cell membrane by inhibiting the activity of ergosterol demethylase in the pathogens, exhibiting strong inhibitory effects against Fragrant Pear Valsa Canker, with an EC_{50} as low as 0.36 mg/L. However, long-term and exclusive use of chemical agents leads to the rapid development of pathogen resistance, and pesticide residues pose potential risks to the environment and human health. Therefore, it is urgent to develop green and sustainable control strategies.

[04] Biogenic fungicides have become a research hotspot due to their environmental compatibility and low tendency to induce resistance. Among them, *Bacillus subtilis*, a common biocontrol agent, exhibits antagonistic activity against a variety of phytopathogenic fungi. Literature reports that a *Bacillus subtilis* single agent has an EC_{50} of 0.89 mg/L against *Valsa mali*. However, its efficacy in preventing and treating Fragrant Pear Valsa Canker remains unclear, and single-agent treatment has the drawbacks of a prolonged action time and insufficient rapid efficacy.

[05] Although a few studies in the prior art have explored the compound of biogenic and chemical agents, an efficient and low-risk agent combination solution specifically targeting Fragrant Pear Valsa Canker is still lacking in systematic screening and validation. In particular, the synergistic effect and optimal ratio of the compound of *Bacillus subtilis* and tebuconazole have not yet been disclosed.

Statement of Invention

[06] To address the aforesaid technical problems, the present invention provides an application of a *Bacillus subtilis* and tebuconazole compound in preventing and treating Fragrant Pear Valsa Canker.

[07] To achieve the aforesaid objective, a fungicidal composition for preventing and treating Fragrant Pear Valsa Canker is provided in the present invention, including an active ingredient *Bacillus subtilis* and tebuconazole, wherein a weight ratio of the *Bacillus subtilis* to the tebuconazole ranges from 1:1 to 1:3.

[08] Further, a compounding weight ratio of the *Bacillus subtilis* to the tebuconazole is 1:1.

[09] Further, a compounding weight ratio of the *Bacillus subtilis* to the tebuconazole is 1:3.

[10] Further, the *Bacillus subtilis* has a viable cell count of 10 billion CFU/g and is formulated as a wettable powder.

[11] Further, the tebuconazole is formulated as a suspension concentrate with a content of 43%.

[12] An application of the aforesaid fungicidal composition in preventing and treating Fragrant Pear Valsa Canker is further provided in the present invention, wherein the Fragrant Pear Valsa Canker is caused by pathogens *Cytospora pyri*, *C. nivea*, or *C. chrysosperma*.

[13] Further, the fungicidal composition controls the disease by inhibiting mycelial growth of the pathogens, and inhibition rates against strains L48, GP21, and YST10 are all above 80%.

[14] Further, the fungicidal composition is prepared into a fungicidal

composition-containing culture medium or a fungicidal composition solution that will be brushed.

[15] Compared with the prior art, the present invention has the following advantages and technical effects:

[16] In the present invention, inhibitory effects of biogenic fungicides against three types of Fragrant Pear Valsa Canker pathogens are determined using a mycelial growth inhibition method, biogenic fungicides with strong inhibitory effects are then selected and compounded with tebuconazole, and synergistic effects of the biogenic fungicide-tebuconazole compound are investigated using a Wadley method. The present invention provides an efficient and low-risk agent combination solution for green prevention and control of Fragrant Pear Valsa Canker, and is of significance in reducing dependence on chemical pesticides.

Brief Description of the Drawings

[17] FIG. 1 illustrates inhibitory effects of different single biogenic fungicides on mycelial growth of Fragrant Pear Valsa Canker pathogens, wherein A: 2 mg/L L48 osthole; B: 2 mg/L GP21 osthole; C: 2 mg/L YST10 osthole; D: 6.7 mg/L L48 *Bacillus subtilis*; E: 5 mg/L GP21 *Bacillus subtilis*; F: 5 mg/L YST10 *Bacillus subtilis*; G: 67 mg/L L48 *Bacillus polymyxa*; H: 67 mg/L GP21 *Bacillus polymyxa*; and I: 67 mg/L YST10 *Bacillus polymyxa*.

[18] FIG. 2 illustrates inhibitory effects of different biogenic fungicides compounded with tebuconazole on mycelial growth of Fragrant Pear Valsa Canker pathogens, wherein A: 1:3 L48 osthole; B: 1:3 GP21 osthole; C: 1:1 L48 *Bacillus subtilis*; D: 1:3 L48 *Bacillus subtilis*; E: 1:3 GP21 *Bacillus subtilis*; F: CK L48; G: CK GP21; and H: CK YST10.

Detailed Description

[19] Example 1

[20] 1 Materials and methods

[21] 1.1 Test materials

[22] Test strains: Existing pathogens of Fragrant Pear Valsa Canker, including *Cytospora pyri*, *C. nivea*, and *C. chrysosperma (pers.) sordida*, preserved at the Green Prevention and Control Laboratory of Tarim University were selected as research subjects, which were designated as L48, GP21, and YST10, respectively.

[23] Biogenic fungicides: Four commonly available biogenic fungicides on the market were selected for the test, as shown in Table 1-1.

[24] Table 1-1 Test agents

	Test agents	Formulation	Supplier
Biogenic fungicide	1 million spores/g *Pythium oligandrum*	Wettable powder (WP)	Biopreparáty, spol. s r. o.
	10 billion CFU/g *Bacillus subtilis*	Wettable powder (WP)	BioWorks, Inc.
	1% osthole	Emulsion in water (EW)	Inner Mongolia Kingbo Biotech Co., Ltd.
	5% allicin	Microemulsion (ME)	Chengdu Newsun Crop Science Co., Ltd.
	500 million/mL *Bacillus polymyxa*	Aqueous solution (AS)	Hubei Province Bacillus Engineering Technology Center
Chemical pesticide	43% tebuconazole	Suspension concentrate (SC)	Jiangxi Longdeng Chemical Co., Ltd.

[25] 1.2 Test methods

[26] 1.2.1 Preparation of a fungicide-containing culture medium

[27] After high-temperature sterilization, a PDA culture medium was cooled to approximately 50°C at room temperature. Fungicide dilutions prepared at various concentrations were added to the culture medium and mixed thoroughly. Then the mixture was quickly poured into Petri dishes after bubble dissipation and left to stand for preparing a fungicide-containing culture medium. A blank control group was also prepared by adding

sterile water of the same volume as the fungicide solution.

[28] 1.3 Determination methods

[29] 1.3.1 Determination of inhibitory effects of different biogenic fungicides on Fragrant Pear Valsa Canker

[30] A mycelial growth rate method was used in this test. The biogenic fungicides were diluted to various concentration gradients using sterile water. Then, 300 μL of each diluted solution was added to 30 mL of sterilized PDA culture medium at approximately 45°C. The mixture was shaken quickly to ensure thorough mixing, and after bubble dissipation, the mixture was poured into each sterilized Petri dish (approximately 10 mL per dish). Each fungicide was tested at more than five concentration gradients, and the PDA culture medium containing sterile water was used as the control. Each concentration gradient was tested in triplicate. Mycelial plugs with a diameter of 5 mm were punched from an edge of Fragrant Pear Valsa Canker fungal colonies pre-cultured for 3 days at 26°C in the dark using a sterilized cork borer, and then were inverted and inoculated in a center of the fungicide-containing medium. After incubation for 3 days at 26°C in the dark, colony diameters were measured using a cross-line method, and a mycelial growth inhibition rate of the fungicide against Fragrant Pear Valsa Canker was calculated based on an inhibition rate formula.

[31] Mycelial growth inhibition rate (%) = $\frac{\text{Control colony diameter}-\text{Treated colony diameter}}{\text{Control colony diameter}-\text{Mycelial plug diameter}}$ (1-1)

[32] Concentrations of the test agents are listed in Table 1-2.

[33] Table 1-2 Inhibitory concentrations of different biogenic fungicides on mycelial growth of Fragrant Pear Valsa Canker pathogens

Fungicide	Determination concentrations for mycelial growth toxicity (mg/L)
Allicin	4.67, 5.12, 12.8, 32, 57, 80, 200
Osthole	0.56, 0.63, 0.67, 0.83, 1.1, 2, 20
Pythium oligandrum	1110, 1250, 1430, 1600, 2000

Bacillus polymyxa (100 million cfu/mL)	5.58, 6.28, 8.38, 16.7, 67
Bacillus subtilis (100 million cfu/mL)	3.3, 4, 5, 6.7, 12.5

[34] Note: The concentrations of these biogenic fungicides were determined based on preliminary tests.

[35] 1.3.2 Determination of inhibitory effects of different biogenic fungicides compounded with tebuconazole on Fragrant Pear Valsa Canker

[36] Single biogenic fungicides with strong inhibitory effects against Fragrant Pear Valsa Canker pathogens identified in preliminary tests (denoted as “a”) were compounded with 43% tebuconazole EC (denoted as “b”) at ratios of 1:1, 1:3, and 3:1 respectively to prepare mixed agents. PDA plates containing compounds at different ratios were prepared. The mycelial growth rate method described in 1.3.1 was adopted, and fungal colonies were incubated under the same conditions for 3 days. Mycelial diameters and mycelial growth inhibition rates were measured, and toxicity regression equations and EC_{50} values of the compounds at different ratios were calculated based on a toxicity determination method for single agents.

[37] Mycelial growth inhibition rate (%) = $\frac{\text{Control colony diameter}-\text{Treated colony diameter}}{\text{Control colony diameter}-\text{Mycelial plug diameter}}$ (1-2)

[38] Compound concentrations of the test agents are listed in Table 1-3.

[39] Table 1-3 Inhibitory concentrations of different biogenic fungicides compounded with tebuconazole on mycelial growth of Fragrant Pear Valsa Canker pathogens

Fungicide	Strain code	Determination concentrations for mycelial growth toxicity					
Osthole (1:1) mg/L	L48	1.438	1.917	2.875	5.750	7.188	14.375
	GP21						
	YST10						
Bacillus polymyxa (1:3) (100 million cfu/mL)	L48	$8x10^4$	$1x10^5$	$1.1x10^5$	$1.3x10^5$	$1.35x10^5$	
	GP21						
	YST10						
Bacillus subtilis	L48	$5x10^4$	$6x10^4$	$8x10^4$	$1x10^5$	$1.55x10^5$	

(1:3) (100 million cfu/mL)	GP21 YST10

[40] Note: Except for osthole, active ingredient concentrations of *Bacillus polymyxa* and *Bacillus subtilis* could not be calculated; therefore, dilution ratios were used instead.

[41] 1.3.3 Evaluation of synergistic effects of compounds

[42] The biological activity and synergistic coefficient of the compounds were evaluated using a Wadley method to calculate co-toxicity coefficient (CTC), and synergistic ratio (SR) was calculated based on the following formula. In the formulas, EC_{50}(th) refers to a theoretical value of the mixed agent, wherein a and b represent percentages of single biogenic fungicides in the compound; EC_{50}(ob) refers to an actual value of the compounds at different ratios; SR indicates an interaction between single agents a and b after mixing, wherein an SR value less than 0.5 indicates an antagonistic effect, an SR value between 0.5 and 1.5 indicates an additive effect, and an SR value greater than 1.5 indicates a synergistic effect.

[43] $EC_{50}(th)= \frac{a+b}{\frac{a}{EC50(A)}+\frac{b}{EC50(B)}}$ (1-3)

[44] $SR=\frac{EC50(th)}{EC50(ob)}$ (1-4)

[45] In the above formulas, a and b represent proportions of single agents in the mixed agent, respectively.

[46] 1.4 Data processing

[47] Test data were processed using Excel software for basic calculations of means and inhibition rates, and SPSS.27 software was used for analysis of variance (ANOVA), toxicity regression equations, and EC_{50} value calculations.

[48] 2 Results and analysis

[49] 2.1 Inhibitory effects of different single biogenic fungicides on mycelial growth of Fragrant Pear Valsa Canker pathogens

[50] Inhibitory effects of different biogenic pesticides on mycelial growth of Fragrant Pear

Valsa Canker pathogens were determined using a mycelial growth inhibition method, as shown in FIG. 2-1. Test results showed that, except for 1 million spores/g *Pythium oligandrum*, the other four biogenic fungicides, including 10 billion CFU/g *Bacillus subtilis*, 500 million CFU/mL *Bacillus polymyxa*, 1% osthole, and 5% allicin, all exhibited a certain degree of inhibitory effects against the mycelial growth of the Fragrant Pear Valsa Canker pathogens with three different pathogenic intensities, as shown in FIG. 2-1. As shown in Table 2-1, concentration ranges were 3.3-12.5 mg/L for 10 billion cfu/g *Bacillus subtilis*, 5.58-67 mg/L for 500 million CFU/mL *Bacillus polymyxa*, and 0.63-20 mg/L for 1% osthole. Based on the analysis of the inhibitory effects of five fungicides on the mycelial growth of three types of Fragrant Pear Valsa Canker pathogens (L48, YST10, and GP21), as shown in Table 2-2, it was found that 10 billion CFU/g *Bacillus subtilis* exhibited an EC_{50} (L48) value of 1.06 mg/L, an EC_{50}(Gp21) value of 2.22 mg/L, and an EC_{50} (YST10) value of 1.02 mg/L; 500 million CFU/mL *Bacillus polymyxa* exhibited an EC_{50} (L48) value of 1.70 mg/L, an EC_{50} (Gp21) value of 4.49 mg/L, and an EC_{50} (Yst10) value of 0.03 mg/L; 1% osthole exhibited an EC_{50} (L48) value of 2.30 mg/L, an EC_{50} (Gp21) value of 2.06 mg/L, and an EC_{50} (Yst10) value of 1.05 mg/L.

[51] Table 2-1 Inhibitory effects of different single biogenic fungicides on mycelial growth of Fragrant Pear Valsa Canker pathogens

Agents	Agent concentration	L48		YST10		GP21	
		Colony diameter / cm	Inhibition rate / %	Colony diameter / cm	Inhibition rate / %	Colony diameter / cm	Inhibition rate / %
Osthole(mg/L)	20	0.87	97.20	0.80	98.63	1.05	95.21
	2	1.80	81.51	1.62	87.44	1.72	86.07
	1.1	4.30	39.50	4.37	49.77	3.78	57.76
	0.83	5.30	22.69	4.65	45.89	7.63	24.43
Osthole (mg/L)	0.67	5.25	23.53	4.57	47.03	6.22	11.42
	0.63	6.05	10.08	5.05	40.41	7.17	5.02
Allicin (mg/L)	80	3.38	62	1.70	86	2.25	71

	57	3.55	60	2.25	79	2.85	63
	32	4.33	48	2.57	74	3.05	59
	12.8	5.17	37	3.20	66	4	43
	5.12	5.78	28	5.28	37	5.6	22
	4.67	6.82	13	6.55	20	5.95	14
	67.0	1.02	96	1.73	86	3.25	65
Bacillus polymyxa	16.7	3.57	60	2.37	77	4.08	54
(100 million	8.38	4.78	44	3.40	63	5.47	35
cfu/mL)	6.28	4.93	42	3.60	60	6.57	20
	5.58	7.83	2	5.80	30	7.65	5
	12.5	1.50	89.19	1.22	93.02	1.47	89.64
	6.7	1.78	85.36	2.07	86.94	2.03	81.98
Bacillus subtilis	5	2.83	71.17	1.67	81.53	2.12	80.86
(100 million	4	3.00	68.92	2.57	74.77	2.25	79.05
cfu/mL)	3.3	4.38	50.23	2.53	75.23	2.48	75.90
	200	2.45	75	1.42	90	2.05	76

[52] Table 2-2 Inhibitory effects of different single biogenic fungicides on mycelial growth of Fragrant Pear Valsa Canker pathogens

Agents	Strain code	EC_{50}	Correlation coefficient r	Toxicity regression equation
Allicin (mg/L)	L48	8.86	0.9671	y=3.5231+0.9527x
	GP21	9.49	0.9538	y=3.5131+1.0401x
	YST10	14.59	0.9517	y=3.7577+1.1805x
Osthole (mg/L)	L48	2.31	0.9789	y=2.2741+2.8581x
	GP21	2.06	0.9369	y=3.3810+2.1364x
	YST10	1.05	0.9686	y=2.2633+1.1014x
Bacillus polymyxa	L48	1.69	0.9317	y=0.7201+2.6813x

(100 million cfu/mL)	GP21	4.49	0.9429	y=1.3164+1.5244x
	YST10	0.03	0.9572	y=2.9279+1.1639x
Bacillus subtilis (100 million cfu/mL)	L48	0.26	0.9327	y=0.1620+7.0137x
	GP21	2.22	0.9814	y=3.2262+0.9163x
Tebuconazole (mg/L)	YST10	0.17	0.9436	y=1.9646+6.3425x
	L48	0.36	0.9635	y=0.5868+9.2790x

[53] 2.2 Determination of inhibitory effects of different biogenic fungicides compounded with 43% tebuconazole on Fragrant Pear Valsa Canker

[54] Compounding at three different ratios was performed in this test. Biogenic fungicides were compounded with tebuconazole at ratios of 1:1, 1:3, and 3:1 under the same dilution concentration multiples. The mycelial growth inhibition method described in 2.1.1 was used to calculate the mycelial growth inhibition rate. According to the test results, the osthole and tebuconazole compound showed a certain degree of inhibitory effects on all three types of Fragrant Pear Valsa Canker pathogens. The *Bacillus subtilis* and tebuconazole compound completely inhibited strain YST10 and showed varying degrees of inhibition on the other two strains. The *Bacillus polymyxa* and tebuconazole compound showed no inhibitory effect on strain GP21.

[55] 2.3 Inhibitory and synergistic effects of different biogenic fungicides compounded with tebuconazole on Fragrant Pear Valsa Canker pathogens

[56] The compounding effects of osthole and tebuconazole are shown in Table 2-3. The compound exhibited additive or synergistic effects on all three strains. Except for L48 at a 1:1 ratio and GP21 at a 3:1 ratio, which showed additive effects, all other compounds showed synergistic effects. The *Bacillus polymyxa* and tebuconazole compound showed no inhibitory effect on GP21, but exhibited complete inhibition on YST10. At a 1:3 ratio, the inhibition rate on L48 reached 91.49%. The *Bacillus subtilis* and tebuconazole compound exhibited complete inhibition on YST10 and achieved inhibition rates of over 80% against L48 and GP21, as shown in FIG. 2-2.

[57] Table 2-3 EC_{50} values and compounding effects on Fragrant Pear Valsa Canker

Strain code	Agent treatment	Toxicity regression equation	EC_{50}	Correlation coefficient r	Synergistic coefficient	Compound effect
	Ph	y=3.8741+2.8581x	1.71	0.9789		
	Py	y=0.5868+9.2790x	0.36	0.9635		
	Ph:Py=1:1	y=3.9746+1.9606x	0.52	0.9942	1.356	Additive
L48	Ph:Py=1:3	y=4.1284+1.4835x	0.59	0.9165	1.620	Synergistic
	Ph:Py=3:1	y=3.3347+0.5705x	0.47	0.9703	2.942	Synergistic
	Ph:Py=3:1	y=3.5961+1.0405x	1.06	0.9367	1.0611	Additive
GP21	Ph:Py=1:1	y=1.7515+2.7248x	0.44	0.9839	1.6128	Synergistic
	Ph:Py=1:3	y=2.2825+2.3857x	0.14	0.9628	2.5790	Synergistic

[58] The application of biogenic fungicides in preventing and treating Fragrant Pear Valsa Canker in the present invention is a new direction in the field of biological control. It also offers a new approach to reducing the use of chemical agents, improving the environment, and mitigating pollution, making it an important step toward long-term development. As only laboratory efficacy tests were performed, the efficacy of disease control in the fields remains to be further verified. To address the issues of environmental pollution and resistance caused by chemical agents, the compound of chemical agents with biogenic fungicides provides a strategy that enhances disease control efficacy while supporting long-term development.