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Research Article

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Screening of disinfectants and their selective toxicity at lower temperature to bursaphelenchus xylophilus and bacteria

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ABSTRACT

Pine wilt disease is caused by a complex of the pine wood nematode (PWN), Bursaphelenchus xylophilus and its associated bacteria. To isolate the aseptic nematode, the sterilizing effect of seven chemicals on PWN and its accompanying bacteria Burkholderia cepacia (strain B619) in a series of concentration and treating time were investigated. B619 could be completely killed by peroxide, sodium hypochlorite, mercuric chloride, malachite green, and gentamycin and couldn't by lysozyme and streptomycin. PWNs could not be disinfected effectively by any of these chemicals. However, The survival rate of PWN decreased as the treatment time and concentration of these disinfectants were increased. Treatments at room temperature (22 °C to 26 °C) or at low temperature (4 °C) improved the eliminating effect of 0.1% mercuric chloride, 3% peroxide, and 4% sodium hypochlorite. Higher PWN survival was observed at low temperatures compared with that at room temperature. The chemicals that most effectively eliminated the bacteria and decreased the PWN survival rate were 3% peroxide, 0.1% mercuric chloride, and 4% sodium hypochlorite. The results suggested that PWN might have a protective effect on its associated bacteria.

Keywords: Bursaphelenchus xylophilus, accompanying bacteria, disinfectants, temperature, Survival of PWN

INTRODUCTION

The pine wilt disease (PWD), which leads to the wilting and subsequent death of infected trees, is one of the main diseases found in conifer forests [1, 2]. PWD is a worldwide threat to pine forests and other forest ecosystems. The pine wood nematode (PWN), Bursaphelenchus xylophilus, was previously thought to be the only pathogenic agent that caused the disease [1, 3-5]. However, surface-sterilized PWNs were reported to lose their pathogenicity [6, 7]. Bacteria were likewise associated with PWNs [8, 9]. Zhao et al. (2000) studied the surface of PWNs with a scanning electron microscope and found bacteria attached to the nematode body surface[10]. Thus, a complex of PWN and its associated pathogenic bacteria was proposed to cause PWD[10]. Consequently, the isolation of aseptic PWNs is important for research on the relationships between these bacteria and PWN.

PWNs are often treated with 0.5% thiomersalate or 3% peroxide, then with 0.5% streptomycin to obtain the aseptic PWNs [12-16]. However, PWNs were reported to still carry bacteria even after these treatments [14, 16]. PWNs that were treated with high concentrations of peroxide (up to 15%) or with a mixture of antibiotics such as penicillin, streptomycin, and gentamycin were found to carry bacteria after the treatment. These results indicated the difficulty and complexity of sterilizing PWNs [17]. The present study aimed to improve currently available sterilization methods for PWNs.

EXPERIMENTAL SECTION

Source and culture of PWN

PWN (B. xylophilus) was isolated from a naturally infected Japanese black pine tree (Pinus thunbergii) in the suburbs of Nanjing (Jiangsu, China). One adult male and one adult female were selected and identified as B. xylophilus, and then both were cultured with the fungus Botrytis cinerea on a single potato sucrose agar (PSA) plate. Subsequently, the plate was incubated until the fungal mycelia were completely consumed. The nematodes were separated from the culture medium by using a Baermann funnel to obtain an aqueous suspension of nematodes for further use.

Acquisition of bacterium accompanying PWN

The B619 strain of Burkholderia cepacia (Palleroni and Holmes, 1981; Yabuuchi et al., 1992), also known as Pseudomonas cepacia (Burkholder, 1950; Palleroni and Holmes, 1981), was isolated from PWNs collected from a tree that died of PWD [13]. This bacterial strain was cultured with nutrient broth (NB). In the subsequent experiments, a bacterial density of 1013 ml-1 was used based on the optical density (OD) value.

Chemicals for the treatment of PWN

The following treatment concentrations for different chemicals were prepared:
(1)peroxide at 1%, 2%, and 3% (w/w);
(2)sodium hypochlorite at 1%, 2%, 3%, and 4% (w/w);
(3)mercuric chloride at 0.01%, 0.05%, 0.1%, and 0.5% (w/w);
(4)malachite green at 0.01%, 0.05%, 0.1%, and 0.5% (w/w);
(5)lysozyme at 10, 20, 30, 40, and 50 U·mg-1;
(6)streptomycin at 0.1, 0.5, 1, and 5 U·mg-1; and
(7) gentamycin at 0.1, 0.5, 1, and 5 U·mg-1.

Elimination of the associated bacteria

PWN and the accompanying bacteria were treated with the abovementioned chemicals at different concentrations and treatment times. The sterilization effect of these chemicals on the bacteria when they were associated with PWNs in the culture, the sterilization effect on pure cultures of the associated bacteria, and the survival rate of the treated PWNs were observed.

A 100 μL solution containing approximately 1000 nematodes were added to 10 ml solutions of the various chemicals with different concentrations. The B619 strain was cultured in NB until the medium became turbid. Then, the medium was centrifuged at 8000 rpm (5581×*g*) and the supernatants were discarded. Sterile water was added to the remaining pellet to obtain the original volume of the suspensions. Finally, 100 μL of the suspension was added to 10 ml of the different concentrations of each chemical solution.

The chemical solutions with either PWNs or the bacteria were incubated on a low-speed shaker at 10 rpm to 20 rpm on the shaker. The treatment times used for the peroxide, sodium hypochlorite, mercuric chloride, and malachite green treatments were 5, 10, 15, and 20 min, whereas the lysozyme, streptomycin, and gentamycin treatments were performed for 0.5, 1.0, 1.5, and 2.0 h at room temperature (22 °C to 26 °C).

After treatment with the respective chemicals, the PWN and bacterial samples were washed in sterile water three times, and then the PWN samples were centrifuged at 2 000 rpm (1400×*g*) and the bacterial samples at 8 000 rpm (5581×*g*). The supernatants were discarded for both types of samples. The remaining pellet of each treated sample was incubated in NB at 25 °C. The sterilization effect on PWN and the eliminating effect on B619 were observed daily until the medium became turbid for up to 9 d. Prior to the incubation of the PWN pellet into the medium, approximately 50 to 100 PWNs were obtained from each treated sample to measure the number of PWNs that survived for the calculation of their survival rate.

Effect of temperature on disinfectant toxicity to PWNs and bacteria

PWNs and their accompanying bacteria were treated with the three disinfectants (4% sodium hypochlorite, 3% peroxide, and 0.1% mercuric chloride) at room temperature (22 °C to 26 °C) and low temperature (4 °C). The effect of the incubation temperature on the disinfectant toxicity to PWNs and their associated bacteria was observed.

Table 1. Sterilizing Effect of the chemincals on PWN and Its Associated Bacteria, as well as PWN Survival after Treatment

Chemical	Treated object	treatment concentration*	A				B				C				D				E			
		treatment time (min)	5	10	15	20	5	10	15	20	5	10	15	20	5	10	15	20	5	10	15	20
Peroxide	PWN	NB turbidity**	+	+	+	+	+	+	+	+	+	+	+	+								
		survival (%)	100	100	100	100	100	100	100	100	100	100	99	99								
	B619	NB turbidity	—	—	—	—	—	—	—	—	—	—	—	—								
Sodium hypochlorite	PWN	NB turbidity	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
		survival (%)	100	100	100	100	100	100	100	95	100	97	84	68	96	89	75	41				
	B619	NB turbidity	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—				
Mercuric chloride	PWN	NB turbidity	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
		survival (%)	100	100	100	100	100	100	100	93	100	100	98	95	100	97	89	72				
	B619	NB turbidity	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—				
Malachite green	PWN	NB turbidity	++	++	++	++	++	++	++	+	++	++	+	+	+	+	+	+				
		survival (%)	100	100	100	100	100	100	100	100	100	100	100	95	100	96	94	91				
	B619	NB turbidity	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—				
Lysozyme	PWN	NB turbidity	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
		survival (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	B619	NB turbidity	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptomycin	PWN	NB turbidity	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++				
		survival (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100				
	B619	NB turbidity	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
Gentamycin	PWN	NB turbidity	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++				
		survival (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100				
	B619	NB turbidity	+	+	+	+	+	+	+	—	+	+	—	—	—	—	—	—				

** Treatment concentration (%); Peroxide (%): A = 1, B = 2, C = 3; Sodium hypochlorite (%): A = 1, B = 2, C = 3, and D = 4; Mercuric chloride (%): A = 0.01, B = 0.05, C = 0.1, and D = 0.5; Malachite green (%): A = 0.01, B = 0.05, C = 0.1, D = 0.5; Lysozyme ($U \cdot mg^{-1}$): A = 10, B = 20, C = 30, D = 40, E = 50; Streptomycin ($U \cdot mg^{-1}$): A = 0.1, B = 0.5, C = 1, D = 5; Gentamycin ($U \cdot mg^{-1}$): A = 0.1, B = 0.5, C = 1, D = 5.*

*** NB turbidity; "—" means the medium is clear and axenic; "+" means that the bacteria are alive and the medium becomes turbid, "++" means that more bacteria are present and the medium becomes turbid more quickly than "+".*

Approximately 1 000 PWNs were treated with the three disinfectant solutions at room temperature and at low temperature conditions for different incubation times. Then, the PWNs were immediately washed in sterile water and added into the NB culture medium. Based on the apparent turbidity of the culture medium, the presence of bacteria on the PWN was observed. Before the PWNs were inoculated into the NB medium, approximately 50 to 100 PWNs were obtained from each treatment sample. The survival states of these isolated PWNs were observed under the microscope. For comparison, pure cultures of the accompanying bacteria were treated according to the same protocol as the PWNs. The treatment time for B619 and the PWNs was 20 min for the room temperature treatments, in which the time interval between observations was 5 min. Approximately 10 min to 90 min was used for the low-temperature treatments, in which the time interval between observations was 10 min. The treatment times that were used to determine the effects of disinfectants on the survival rate of PWNs were 3.25 h for the room temperature treatments, in which the time interval between observations was 0.25 h, and 12.5 h for the low temperature treatments, in which the time interval between observations was 0.5 h.

RESULTS AND DISCUSSION

Effects of different disinfectants on PWNs and B619

The sterilization effect on PWNs and the accompanying bacterium (strain B619) were based on the turbidity of the NB medium in which the treated samples were cultured. The toxicity level of each disinfectant on PWNs was based on the survival rate of the PWNs treated with these chemicals (disinfectant, antibiotic, etc.). The results are summarized in Table 1.

Table 1 shows that lysozyme, streptomycin, and gentamycin did not influence the survival rate of PWNs. B619 could be killed by gentamycin at a high concentration (>1%) but remained unaffected by lysozyme and streptomycin at different concentrations or by gentamycin at a low concentration (≦0.5%). PWNs could not be completely sterilized by these chemicals. That is, the sterilizing capacity of the three chemicals is not strong enough to prevent the turbidity of the culture medium within a short period of time. The experimental results proved that the aseptic PWNs obtained with the antibiotics prior were rigorous.

Table 1 also shows that peroxide, sodium hypochlorite, mercuric chloride, and malachite green could effectively kill the associated bacteria when these were cultured alone even at low concentration. However but PWNs could not be completely sterilized by these disinfectants. Instead, the survival rate of PWNs declined as the disinfectant concentration and the treatment time were increased. The chemicals that influenced PWN survival, in the order of strongest toxicity to the weakest, were as follows: peroxide, mercuric chloride, malachite green, and sodium hypochlorite. Based on the results of the eliminating effect on bacteria and the influence on PWN survival, the following disinfectants were chosen for further experiments: peroxide, mercuric chloride, and sodium hypochlorite.

Effect of temperature on disinfectant toxicity to PWNs and bacteria

PWNs and their associated bacteria were treated with 0.1% mercuric chloride, 3% peroxide, and 4% sodium hypochlorite at either room temperature or low temperature. The sterilizing effects on each sample that was tested in NB are presented in Table 2.

Table 2. Sterilizing Effect of Disinfectants on PWN and Its Associated Bacteria at Different Temperatures*

Disinfectant		low temperature									room temperature				
	treatment time (min)	10	20	30	40	50	60	70	80	90	5	10	15	20	25
3% peroxide	PWN	+ 2.5	+ 2.5	+ 4.5	+ 2.5	6.5	—	+ 1		+ 4.5	+ 6.5	+ 6.5	1.5	2.5	6.5
	B619	—	—	—	—	—	—	—	—	—	—	—	—		
0.1% mercuric chloride	PWN	+ 2	+ 2.5	+ 2.5	+ 2.5	+ 2	+ 2.5	+ 2.5	2.5	+ 2.5		+ 3.5	1.5	+ 2	3.5
	B619	—	—	—	—	—	—	—	—	—	—	—	—	—	
4% sodium hypochlorite	PWN	+ 0.5	+ 0.5	+ 0.5	+ 1	+ 1	+ 0.5	+ 1	+ 1	+ 1	+ 1	+ 1.5	1.5	1.5	
	B619	—	—	—	—	—	—	—	—	—	—	—	—	—	

** NB turbidity: A blank space means there is no available data; "—" means that the medium is clear and axenic; "+" means that the bacteria are alive and the medium becomes turbid. The number after "+" indicates the number of days from inoculation to turbidity.*

PWN survival after treatment with 0.1% mercuric chloride, 3% peroxide, and 4% sodium hypochlorite at room temperature and at low temperature are illustrated in Figs. 1 and 2, respectively.

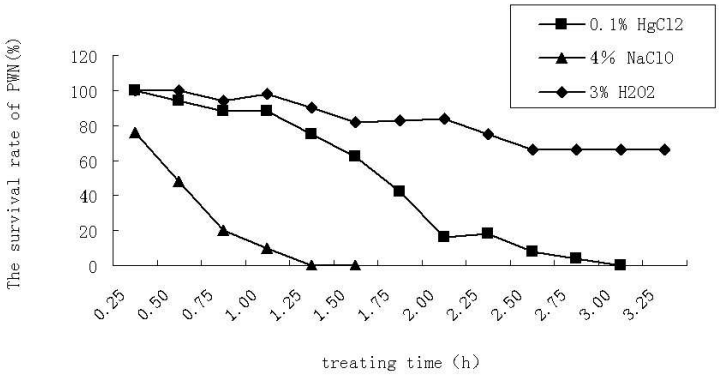

Fig. 1. Survival Rate of PWNs Treated with Disinfectants at Room Temperature (22 °C to 26 °C)

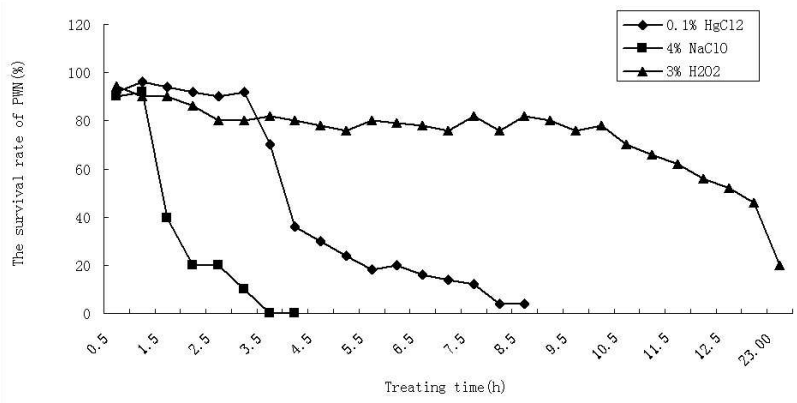

Fig. 2. Survival Rate of PWNs Treated with Three Disinfectants at Low Temperature (4 °C)

Table 2 shows that B619 alone can be completely sterilized by 0.1% mercuric chloride, 3% peroxide, and 4% sodium hypochlorite within 10 min at low temperatures and within 5 min at room temperature.

However, Table 2 shows that aseptic PWNs could not be obtained because the nematodes could not be completely sterilized by the three disinfectants even after 1.5 h of treatment at low temperature or after 25 min of treatment at room temperature either. Likewise, the sterilizing effect did not increase with the increased treatment times. Despite the inability of the chemicals to produce completely aseptic PNWs, the NB medium inoculated with the PWNs that were treated with 3% peroxide did not become turbid after a long period of time (for example, 6.5 d). These results indicated that the concentration of live bacteria on the PWNs approached zero and the nematode might have a protective effect on its associated bacteria.

The results given in Table 2 show that in terms of sterilizing capability, 3% peroxide was the strongest, followed by 0.1% mercuric chloride and 4% sodium hypochlorite. These conclusions were based on the length of time that turbidity was prevented in the NB medium.

Figs. 1 and 2 showed that the PWNs treated with the disinfectants at low temperature had much longer survival times than those treated at room temperature. The order of the increasing PWN survival rate in the samples treated with the three disinfectants at low temperature is 3% peroxide, 0.1% mercuric chloride, and 4% sodium hypochlorite. The same trend was observed for the samples that were treated at room temperature.

Peroxide and sodium hypochlorite are strong oxidants that can cause great damage on biomolecules (protein, lipids, polysaccharide, etc.). Mercuric chloride is toxic to cells. These disinfectants have selective activity because the mucosa and stratum corneum on the surface of animals, which are absent in bacterial cells, could protect animals from damage or virus of the disinfectants. Thus, peroxide and sodium hypochlorite are commonly used disinfectants on animal and plant tissues[18]. Therefore, 0.1% mercuric chloride, 3% peroxide, and 4% sodium hypochlorite are recommended as sterilization agents for PWNs. Inspite of these, the nematodes couldn't treated

with them longer because of the nematodes' short live time especially at room temperature.

We treated PWNs with disinfectants at lower temperature for the first time and increased PWN survival rate which was good much at experiments especially as inoculating as a energetic pathogen. The higher survival rate of PWNs at low temperatures might be attributed to the inability of the nematodes to swallow the disinfectants at low temperatures. The nematodes may also enter a state of suspended animation at low temperatures, thereby decreasing their energy consumption and preventing damage to the nematode tissues and organs. When the PWNs were treated with disinfectants at room temperature (22 °C to 26 °C), the animals were observed to struggle violently upon exposure to the disinfectants.

CONCLUSION

Lysozyme, streptomycin, and gentamycin were unreliable to eliminate PWNs for they could not kill the bacteria completely although they did not influence the survival rate of treated PWNs. Peroxide, sodium hypochlorite, mercuric chloride, and malachite green could effectively kill the associated bacteria alone. According to sterilizing effect and the survival rate of treated nematodes, 4% sodium hypochlorite, 3% peroxide, and 0.1% mercuric chloride could be used to eliminate PWNs. Althougu aseptic PWNs could not be obtained, the concentration of live bacteria on (or in) the sterilized PWNs approached zero and it is such a result that suggested us that the nematode might have a some protective effect on its associated bacteria. Lower temperature could increase treated PWNs' survival rate which was good much at experiments especially as inoculating as a energetic pathogen.

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