Efficacy of a copper-based bactericide in controlling bacterial blight of grapevines caused by *Xylophilus ampelinus*

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Abstract

We investigated the efficacy of a microbial copper agent to protect against bacterial blight of grapevine caused by *Xylophilus ampelinus* from 2012 to 2014 in Hokkaido, Japan. A solution of the basic copper wettable powder sulfate was sprayed at 10-day intervals in two processing plots, using two application protocols: seven rounds of application immediately after leaf development and three or four applications at the initial onset of the disease. Due to the low disease incidence for the duration of the study, symptoms were only observed on leaves, not on spikes or fruits. The disease incidence in plots that were treated at the initial stage of the disease was significantly lower than in untreated plots. This protective effect improved as the number of applications was increased. After overwintering of these vines, *X. ampelinus* was detected in the axillary buds of untreated vines, but it was seldom detected in vines treated at the initial onset of the disease. Thus, the copper agent controlled the extent of infection during the year of treatment and reduced the quantity of bacteria surviving overwintering. Our findings indicate that three or four applications of the agent at the initial stage of the disease should be effective to prevent bacterial blight of grapevines.

Key words: bacterial blight, copper agent, Meta-analysis,

43 overwintering, *Xylophilus ampelinus*

44

Introduction

The gram-negative proteobacterium *Xylophilus ampelinus* (Panagopoulos 1969; Willems et al. 1987) causes blight in its only known host, grapevine (*Vitis vinifera*) (Panagopoulos, 1987). Cool and moist weather conditions are most favorable to the growth of the pathogen (López et al. 1987). The first Japanese outbreak of the disease occurred in 2009 in Hokkaido Prefecture (Shinmura et al. 2012), which is characterized by a cool and moist climate, and diseased plants were distributed throughout the vineyard (A. Shinmura, personal communication). The outbreak led to reduced sugar content in the fruits and withered leaves. Necrosis of the flower and fruits further reduced yields. The symptoms were originally confused with that of dead arm of grapevine caused by *Phomopsis viticola,* a filamentous fungal pathogen that is abundant in Hokkaido. Therefore, almost all grape producers applied fungicide (Benomyl), which failed to control the disease, leading to the discovery of the causal bacterium (Shinmura et al. 2012). *Xanthomonas arboricola* is the only previously identified bacterial pathogen on grapevine reported in Japan. Because the infection caused only mild leaf and fruit spotting (Sawada et al. 2011), bacterial control agents had almost never been used. Copper-containing agents are the only chemicals suitable for anti-bacterial treatment of grapevines.

Previous studies on infections in European vineyards revealed that *X. ampelinus* invades the internal tissues of grapevine. Therefore, copper agents can only be used as preventive treatment against bacteria; they prevent external contamination and, in most cases, the appearance of symptoms.

The importance of the epiphytic phase in disease spread and development of symptoms has been outlined by Grall and Manceau (2003). In contrast to the European studies, in Hokkaido, *X. ampelinus* was not found within, but only on, the underside surface of the bract and wool following overwintering (Komatsu and Kondo, 2015). Therefore, copper agents may be effective for controlling the disease in Hokkaido. The aim of the present study was to examine the effect of basic copper wettable powder sulfate, an antibacterial copper agent, in controlling bacterial blight of grapevines. The effect on the overwintering survival rate of the bacterium was also investigated.

Material and Methods

Antibacterial treatment in vineyards

From 2012 to 2014, spray treatments and observations were done in three commercial vineyards (ridge width, 2.5 m; root distance, 2 m; and unilateral horizontal cordon with spur pruning shaping): vineyard A in Yoichi town where cv. Kerner was planted in 1991 (field size 0.5 ha), vineyard B in Yoichi town

where Kerner was planted in 1995 (field size 0.36 ha), and vineyard C in Furano city where cv. Zweigeltrebe was planted in 1999 (field size 0.3 ha). Each vineyard was severely diseased with bacterial blight in 2009. Because this study was in commercial fields, only registered agricultural chemicals could be tested. Among the available chemicals, only the copper agent was expected to have any effect on the bacterium, so we chose this chemical. Basic copper wettable powder sulfate (content, 58.0%; Z-Bordeaux, Nihon Nohyaku Co., Tokyo, Japan) was dissolved in water to a final concentration of 0.2% (w/v), and calcium carbonate wettable powder (Clef-non; Shiraishi Calcium Ltd., Tokyo, Japan) was added to a final concentration of 1% (w/v) to reduce chemical damage by the copper compound to the leaves. The copper sulfate solution was sprayed using a shoulder-type power sprayer at approximately 10-day intervals in two processing plots as follows: plot 1, application began immediately after leaf development (late-May to mid-June) and was repeated seven times so that the initial stage of the disease always occurred within the treatment period; plot 2, plants were treated three or four times, again with the initial stage of the disease in the spray period (Table 1). The only exception to the protocol was vineyard C in 2012; plot 1 never displayed any initial disease during the test period. In addition to the copper

sulfate solution, standard controls for other diseases were applied; however, the use of chemicals containing copper was avoided. In mid-September, leaves were observed and scored as healthy (absence of spotting) or diseased (presence of spotting). After all the leaves had fallen in October, excess branches were pruned according to standard procedures. The grape plants then passed the winter covered in snow.

Data analysis

Disease incidence in each plot was compared with that of the untreated plot (Fig. 1). All plots had two replications that were arranged at random. Because the experiments were done in different locations and years, on different cultivars, with a different number of leaves examined in each experiment, a meta-analysis of the disease control efficiency of each plot was performed using the fixed-effects method. Meta-analysis is a statistical technique used to collate findings from a number of independent studies. Suppressive effects have been expressed in terms of the integrated risk ratio (DerSimonian and Kacker 2007; DerSimonian and Laird 1986; Kawaguchi et al. 2014; Madden and Paul 2011; Rosenberg et al. 2004; Tango 2002). The meta-analysis was performed using EZR (Kanda, 2013), the graphical user interface for R software version 2.1-2 (R Foundation for Statistical Computing, Vienna, Austria).

Pathogen DNA detection by specific polymerase chain reaction (PCR)

After overwintering, axillary buds were collected to test for the presence of *X. ampelinus*; three buds were collected from 20 vines from each replication before sprouting, from April to May. Each bud was softened by immersion in 100 µL of Tris-EDTA (TE) buffer, and then homogenized using a sterilized mortar and pestle. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Amplification reactions consisted of 1 µL of template DNA and 24 µL of reaction mixture containing 1 µM of each primer (XaTS1, 5′-TGCGTAGTTCAACACCAAAGT-3′; XaTS2, 5′-TATGACCCTCTTTCCACCAGC-3′), 1.25 units of *Ex Taq* DNA polymerase (Takara Bio; Shiga, Japan), 200 µM dNTP mixture (Takara Bio), and 1× PCR buffer with $MgCl_2$ (Takara Bio). Amplification conditions were as follows: initial denaturation at 94 °C for 5 min followed by 40 cycles of 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 45 s with a final extension at 72 °C for 8 min. The PCR products were subjected to 1.5% (w/v) agarose gel electrophoresis for 30 min at 100 V, and the amplicons were detected by staining the gels with 0.01% (v/v) GelRed (Wako, Tokyo, Japan).

Results

Controlling bacterial blight with copper agents in vineyard experiments

In 2012 and 2014, initial symptoms of infection were observed on leaves after mid-August (Table 1). In 2013, although symptoms were observed on some leaves before blooming, the progress of the disease was hampered. As in 2012 and 2014, in 2013, the greatest increase in disease was observed after mid-August (Table 1). The spikes and fruits displayed no symptoms of infection in any observation, because of low overall disease incidence. Because plot 1 of vineyard C never showed any symptoms during the spraying period in 2012, data from this time-point were excluded from the meta-analysis. In plot 1, the risk ratio (RR) varied from 0.10 to 0.19, and the integrated RR (IRR) was 0.12 (95% confidence interval [CI], 0.09–0.17; $P < 0.0001$), indicating that disease incidence was reduced significantly by the treatment. In plot 2, the RR varied from 0.30 to 0.59, and the IRR was 0.38 (95% CI, 0.33–0.45; $P < 0.0001$), indicating that disease incidence was reduced significantly; the IRR of plot 1 was lower than that of plot 2.

Detection of *X. ampelinus* DNA by specific PCR

PCR results for the presence of *X. ampelinus* in the axillary buds after overwintering are summarized in Table 2 and Fig. 2.

Bacterial DNA was detected in 3.3–45.0% of the plants in the untreated plot. However, no bacterial DNA was detected in plots 1 (7 applications) or 2 (3–4 applications).

Discussion

Grapevine blight caused by bacterial infection has reduced crop yields for many years in European vineyards. Preventive measures, including removing infected branches, pruning in dry conditions, and preventing bacterial contamination from pruning tools, are generally carried out. Panagopolos (1987) reported that 13 types of chemical agents tested (copper oxychloride, sodium arsenite, 4,6-dinitro-*o*-cresol, streptomycin, oxytetracycline, tetramycin, kasugamycin, vancomycin, cycloheximide, thiosemicarbazone, 8-hydroxyquinoline benzoate, fentiazon, phenazine 5-oxide) were not effective for disease control after four applications. Bordeaux mixture and dichlorofen were ineffective in Italy (Grasso et al. 1979). López et al. (1987) tested various copper compounds for antibacterial effects, but did not observe any significant protection, probably because of a drought in the year of the study. Despite the widespread belief that chemicals cannot prevent blight, the current study presents evidence for the protective effect of copper wettable powder sulfate. We hypothesize that chemical agents are probably not effective against bacteria that invade inner organs, which could

explain the contradictory results obtained in Europe and Japan (in this study). In France, it was reported that *X. ampelinus* was maintained within the organ and that sap and aged vascular bundles were important reservoirs of inoculum (Grall et al. 2005). In contrast, Komatsu and Kondo (2015) reported that propagules of *X. ampelinus* were not likely to survive in plant tissues or sap throughout the year in Hokkaido because the bacterium can be found only on the underside surfaces of the bract and bud wool after overwintering. In addition, we speculated that the bacteria spread mainly along the surface of the vine to the branches, leaves, or spikes by water or wind, but not within the plant. The data presented in this study are consistent with this hypothesis and demonstrate that the copper agent was in contact with the bacteria on plant surfaces and suppressed growth and spread.

In 2011, occurrence of the disease in the leaves was restrained by seven applications of copper agent, and decline of fruit yield was restrained in Yoichi upon treatment (Shinmura, unpublished data). The disease causes symptomatic with changes in the environment, but the bacteria spread before the symptoms begin to appear. Multiple applications of copper agent are thought to inhibit the spreading phase. However, continuous monitoring in commercial vineyards is difficult, considering the time and labor required. Therefore, producers prefer using more advanced

control methods that involve fewer applications of the copper agent. Although disease incidence was low, and only mild symptoms were observed on leaves, the protective effect conferred by treatment with the copper agent was apparent (Fig. 1). Although these effects were enhanced when the number of applications was increased, sufficient protection was achieved with 3–4 applications, when applied at the initial stage of disease.

Initially, the flowering period was thought to represent the optimum timing to control the disease. However, the findings in 2012 and 2014 revealed that the disease is not always initiated at flowering period. These results indicated that the optimum timing for control is the first observation of the disease, not the growth stage of grapevine. Our results suggest that application of the copper agent three to four times, timed with the initial stage of disease, can control crop damage by *X. ampelinus,* except in cases of a large outbreak.

In this study, we only evaluated damage to leaves. However, during later stages, severe infection leads to necrosis of spikes and fruits, resulting in reduced yield. To avoid economic losses in cases of widespread blight, preventing necrosis of spikes and fruits is important. Because this study did not investigate whether these treatment protocols can prevent fruit decay in the

late phases of growth, it will be necessary to study more severe infection. A promising result from this study was the demonstration that the application of the copper agent both controlled the disease emergence in the present year and reduced the quantity of bacteria that survived the winter (Table 2).

In Hokkaido, although the bacterium was first identified in 2009 (Shinmura et al., 2012), the plants may have been infected several years earlier without showing any symptoms. Because the average amount of rain was higher (131%) during the summer, from June to August in 2009, and the temperatures were lower (−0.6 °C) than usual, a burst of bacterial growth may have induced symptoms. Although recent incidence of disease has been low, the bacteria may spread widely without the plants showing symptoms of infection, priming fields for widespread outbreaks in the future. Because Hokkaido historically has cool summers every few years, the possibility of encountering suitable conditions in the future for disease outbreak is high. For preventing such an outbreak, it is necessary to keep the density of the pathogen in the vineyard low. Our findings indicate that populations of *X. ampelinus* can be controlled by annual application of the copper agent.

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337 **Figure Legends**

338 Fig 1. Integrated evaluation based on a meta-analysis of the
339 effects of bactericide application of a copper agent on bacterial
340 blight of grapevine in nine trials. The center of each symbol
341 marks the value of the integrated risk ratio (IRR). Each gray
342 square marks the value of the risk ratio (RR), and the size of the
343 square indicates how data for each trial were weighted. The
344 horizontal line indicates 95% confidence interval. The dotted
345 vertical line represents the mean RR. Plot 1, application began
346 immediately following leaf development and was repeated seven
347 times so that treatment coincided with the initial stage of the
348 disease; plot 2, plants were treated three or four times,
349 coinciding with the initial stage of the disease.

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351 Fig 2. Results of polymerase chain reaction (PCR) for detecting
352 *Xylophilus ampelinus* in the buds of grapevines without copper
353 (a) treatment or with four applications of copper (b) in 2013 in
354 vineyard 3 after overwintering in the spring of 2014.
355 Lanes: 1, 100-bp ladder; 2, *X. ampelinus* (129 bp, positive
356 control); 3–23, buds, (panel a) no copper, (panel b), copper
357 treatment. No bacteria were detected from treated samples.

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Table 1 Treatment profiles for this study

Year	Vineyard	Plot[a]	No. of grapevines	No. of sprays	Dates of applications							Flowering date	Date of initial disease
2012	A	1	19	7	28-May	18-Jun	3-Jul	17-Jul	24-Jul	2-Aug	15-Aug	3-Jul	15-Aug
		Untreated	20	0	-	-	-	-	-	-	-		
	B	1	17	7	28-May	18-Jun	3-Jul	17-Jul	24-Jul	2-Aug	15-Aug	3-Jul	15-Aug
		Untreated	16	0	-	-	-	-	-	-	-		
	C	1	20	7	31-May	11-Jun	19-Jun	29-Jun	10-Jul	20-Jul	30-Jul	6-Jul	12-Aug
		Untreated	20	0	-	-	-	-	-	-	-		
2013	A	1	20	7	11-Jun	24-Jun	5-Jul	16-Jul	2-Aug	13-Aug	22-Aug	8-Jul	5-Jul
		2	20	4		24-Jun	5-Jul	16-Jul	2-Aug				
		Untreated	20	0	-	-	-	-	-	-	-		
	B	1	18	7	11-Jun	24-Jun	5-Jul	16-Jul	29-Jul	13-Aug	22-Aug	8-Jul	5-Jul
		2	18	4		24-Jun	5-Jul	16-Jul	29-Jul				
		Untreated	17	0	-	-	-	-	-	-	-		
	C	2	18	4	17-Jun	28-Jun	8-Jul	17-Jul	17-Jun			7-Jul	28-Jun
		Untreated	20	0	-	-	-	-	-	-	-		
2014	B	2	21	3				6-Aug	15-Aug	25-Aug		30-Jun	19-Aug
		Untreated	15	0	-	-	-	-	-	-	-		
	C	2	20	3				8-Aug	18-Aug	29-Aug		30-Jun	18-Aug
		Untreated	20	0	-	-	-	-	-	-	-		

[a] Plot 1: First spray began immediately after leafing for seven total sprays to encompass the date of initial disease (i.e., first observation); plot 2: plants were treated three or four times to encompass date of initial disease.

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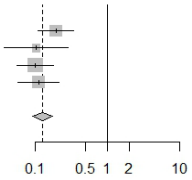
Field trial		Plot 1 Diseased leaves	Plot 1 Total leaves	Plot 1 %	Untreated Diseased leaves	Untreated Total leaves	Untreated %	RR	95% CI	Weight (%)
2012	A	14	5700	0.25	77	6000	1.28	0.19	[0.11-0.34]	23.3
2012	B	4	5100	0.08	37	4800	0.77	0.10	[0.04-0.29]	11.8
2013	A	12	1000	1.20	121	1000	12.10	0.10	[0.06-0.18]	37.5
2013	B	10	900	1.11	86	850	10.12	0.11	[0.06-0.21]	27.3
Integrated risk ratio (p < 0.0001)								0.12	[0.09-0.17]	100

Heterogeneity: I-squared = 2.5%, tau-squared = 0.003, p = 0.38



Field trial		Plot 2 Diseased leaves	Plot 2 Total leaves	Plot 2 %	Untreated Diseased leaves	Untreated Total leaves	Untreated %	RR	95% CI	Weight (%)
2013	A	53	1000	5.30	121	1000	12.10	0.44	[0.32-0.60]	22.4
2013	B	35	900	3.89	86	850	10.12	0.38	[0.26-0.56]	16.4
2013	C	58	900	6.44	212	1000	21.20	0.30	[0.23-0.40]	37.2
2014	B	51	1050	4.86	62	750	8.27	0.59	[0.41-0.84]	13.4
2014	C	17	980	1.73	58	1000	5.80	0.30	[0.18-0.51]	10.6
Integrated risk ratio (p < 0.0001)								0.38	[0.33-0.45]	100

Heterogeneity: I-squared = 58.7%, tau-squared = 0.0462, p = 0.0

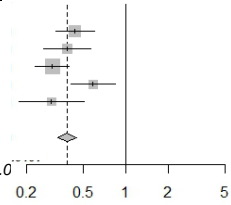


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Table 2 Frequency of PCR detection of DNA specific for *Xylophilus ampelinus* from axillary buds of overwintered grapevines

Plot	Frequency of detection					
	Vineyard, 2012			Vineyard, 2013		
	A	B	C	A	B	C
1	0/60	0/60	0/60	0/60	0/60	nt
2	nt	nt	nt	0/60	0/60	0/60
Untreated	13/60	5/60	24/60	2/60	25/60	27/60

For each test, 3 buds were collected from each of 20 vines before sprouting (April –May 2012 and 2013) and tested separately; nt = not tested

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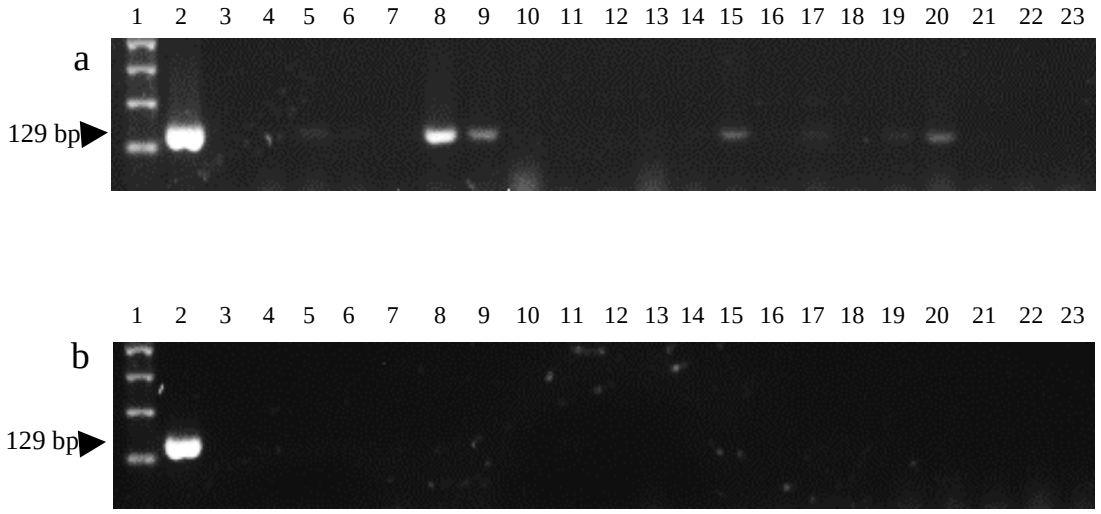
369 Fig. 2



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