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

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19

20 **Abstract**

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

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

43 overwintering, *Xylophilus ampelinus*

44

45 **Introduction**

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

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

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

84 **Material and Methods**

85 **Antibacterial treatment in vineyards**

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

91 where Kerner was planted in 1995 (field size 0.36 ha), and
92 vineyard C in Furano city where cv. Zweigeltrebe was planted in
93 1999 (field size 0.3 ha). Each vineyard was severely diseased
94 with bacterial blight in 2009. Because this study was in
95 commercial fields, only registered agricultural chemicals could
96 be tested. Among the available chemicals, only the copper agent
97 was expected to have any effect on the bacterium, so we chose
98 this chemical. Basic copper wettable powder sulfate (content,
99 58.0%; Z-Bordeaux, Nihon Nohyaku Co., Tokyo, Japan) was
100 dissolved in water to a final concentration of 0.2% (w/v), and
101 calcium carbonate wettable powder (Clef-non; Shiraishi Calcium
102 Ltd., Tokyo, Japan) was added to a final concentration of 1%
103 (w/v) to reduce chemical damage by the copper compound to the
104 leaves. The copper sulfate solution was sprayed using a
105 shoulder-type power sprayer at approximately 10-day intervals
106 in two processing plots as follows: plot 1, application began
107 immediately after leaf development (late-May to mid-June) and
108 was repeated seven times so that the initial stage of the disease
109 always occurred within the treatment period; plot 2, plants were
110 treated three or four times, again with the initial stage of the
111 disease in the spray period (Table 1). The only exception to the
112 protocol was vineyard C in 2012; plot 1 never displayed any
113 initial disease during the test period. In addition to the copper

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

121 **Data analysis**

122 Disease incidence in each plot was compared with that of the
123 untreated plot (Fig. 1). All plots had two replications that were
124 arranged at random. Because the experiments were done in
125 different locations and years, on different cultivars, with a
126 different number of leaves examined in each experiment, a
127 meta-analysis of the disease control efficiency of each plot was
128 performed using the fixed-effects method. Meta-analysis is a
129 statistical technique used to collate findings from a number of
130 independent studies. Suppressive effects have been expressed in
131 terms of the integrated risk ratio (DerSimonian and Kacker
132 2007; DerSimonian and Laird 1986; Kawaguchi et al. 2014;
133 Madden and Paul 2011; Rosenberg et al. 2004; Tango 2002). The
134 meta-analysis was performed using EZR (Kanda, 2013), the
135 graphical user interface for R software version 2.1-2 (R
136 Foundation for Statistical Computing, Vienna, Austria).

137 **Pathogen DNA detection by specific polymerase chain reaction**
138 **(PCR)**

139 After overwintering, axillary buds were collected to test for the
140 presence of *X. ampelinus*; three buds were collected from 20 vines
141 from each replication before sprouting, from April to May. Each
142 bud was softened by immersion in 100 μ L of Tris-EDTA (TE)
143 buffer, and then homogenized using a sterilized mortar and
144 pestle. DNA was extracted using the DNeasy Plant Mini Kit
145 (Qiagen, Hilden, Germany) according to the manufacturer's
146 instructions. Amplification reactions consisted of 1 μ L of
147 template DNA and 24 μ L of reaction mixture containing 1 μ M of
148 each primer (XaTS1, 5'-TGCGTAGTTCAACACCAAAGT-3';
149 XaTS2, 5'-TATGACCCTCTTTCCACCAGC-3'), 1.25 units of *Ex*
150 *Taq* DNA polymerase (Takara Bio; Shiga, Japan), 200 μ M dNTP
151 mixture (Takara Bio), and 1 \times PCR buffer with MgCl₂ (Takara
152 Bio). Amplification conditions were as follows: initial
153 denaturation at 94 °C for 5 min followed by 40 cycles of 94 °C for
154 30 s, 60 °C for 45 s, and 72 °C for 45 s with a final extension at
155 72 °C for 8 min. The PCR products were subjected to 1.5% (w/v)
156 agarose gel electrophoresis for 30 min at 100 V, and the
157 amplicons were detected by staining the gels with 0.01% (v/v)
158 GelRed (Wako, Tokyo, Japan).

159

160 Results

161 Controlling bacterial blight with copper agents in vineyard 162 experiments

163 In 2012 and 2014, initial symptoms of infection were observed
164 on leaves after mid-August (Table 1). In 2013, although
165 symptoms were observed on some leaves before blooming, the
166 progress of the disease was hampered. As in 2012 and 2014, in
167 2013, the greatest increase in disease was observed after
168 mid-August (Table 1). The spikes and fruits displayed no
169 symptoms of infection in any observation, because of low overall
170 disease incidence. Because plot 1 of vineyard C never showed any
171 symptoms during the spraying period in 2012, data from this
172 time-point were excluded from the meta-analysis. In plot 1, the
173 risk ratio (RR) varied from 0.10 to 0.19, and the integrated RR
174 (IRR) was 0.12 (95% confidence interval [CI], 0.09–0.17; $P <$
175 0.0001), indicating that disease incidence was reduced
176 significantly by the treatment. In plot 2, the RR varied from 0.30
177 to 0.59, and the IRR was 0.38 (95% CI, 0.33–0.45; $P <$ 0.0001),
178 indicating that disease incidence was reduced significantly; the
179 IRR of plot 1 was lower than that of plot 2.

180 Detection of *X. ampelinus* DNA by specific PCR

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

183 Bacterial DNA was detected in 3.3–45.0% of the plants in the
184 untreated plot. However, no bacterial DNA was detected in plots
185 1 (7 applications) or 2 (3–4 applications).

186 **Discussion**

187 Grapevine blight caused by bacterial infection has reduced crop
188 yields for many years in European vineyards. Preventive
189 measures, including removing infected branches, pruning in dry
190 conditions, and preventing bacterial contamination from pruning
191 tools, are generally carried out. Panagopolos (1987) reported that
192 13 types of chemical agents tested (copper oxychloride, sodium
193 arsenite, 4,6-dinitro-*o*-cresol, streptomycin, oxytetracycline,
194 tetracycline, kasugamycin, vancomycin, cycloheximide,
195 thiosemicarbazone, 8-hydroxyquinoline benzoate, fentiazon,
196 phenazine 5-oxide) were not effective for disease control after
197 four applications. Bordeaux mixture and dichlorofen were
198 ineffective in Italy (Grasso et al. 1979). López et al. (1987) tested
199 various copper compounds for antibacterial effects, but did not
200 observe any significant protection, probably because of a drought
201 in the year of the study. Despite the widespread belief that
202 chemicals cannot prevent blight, the current study presents
203 evidence for the protective effect of copper wettable powder
204 sulfate. We hypothesize that chemical agents are probably not
205 effective against bacteria that invade inner organs, which could

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

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

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

237 Initially, the flowering period was thought to represent the
238 optimum timing to control the disease. However, the findings in
239 2012 and 2014 revealed that the disease is not always initiated
240 at flowering period. These results indicated that the optimum
241 timing for control is the first observation of the disease, not the
242 growth stage of grapevine. Our results suggest that application
243 of the copper agent three to four times, timed with the initial
244 stage of disease, can control crop damage by *X. ampelinus*, except
245 in cases of a large outbreak.

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

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

257 In Hokkaido, although the bacterium was first identified in
258 2009 (Shinmura et al., 2012), the plants may have been infected
259 several years earlier without showing any symptoms. Because
260 the average amount of rain was higher (131%) during the
261 summer, from June to August in 2009, and the temperatures
262 were lower ($-0.6\text{ }^{\circ}\text{C}$) than usual, a burst of bacterial growth may
263 have induced symptoms. Although recent incidence of disease
264 has been low, the bacteria may spread widely without the plants
265 showing symptoms of infection, priming fields for widespread
266 outbreaks in the future. Because Hokkaido historically has cool
267 summers every few years, the possibility of encountering
268 suitable conditions in the future for disease outbreak is high. For
269 preventing such an outbreak, it is necessary to keep the density
270 of the pathogen in the vineyard low. Our findings indicate that
271 populations of *X. ampelinus* can be controlled by annual
272 application of the copper agent.

273

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281 Furano-city.

282

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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.

350

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.

358

359

Table 1 Treatment profiles for this study

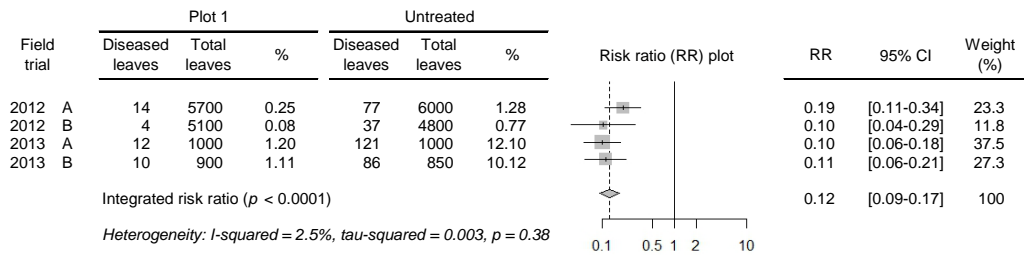
Year	Vineyard	Plot ^a	No. of grapevines	No. of sprays	Dates of applications							Flowering date	Date of initial disease
2012	A	1	19	7	28-May	18-Jun	3-Jul	17-Jul	24-Jul	2-Aug	15-Aug	3-Jul	15-Aug
		Untreated	20	0	-	-	-	-	-	-	-		
	B	1	17	7	28-May	18-Jun	3-Jul	17-Jul	24-Jul	2-Aug	15-Aug	3-Jul	15-Aug
		Untreated	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	2	18	4	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
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.

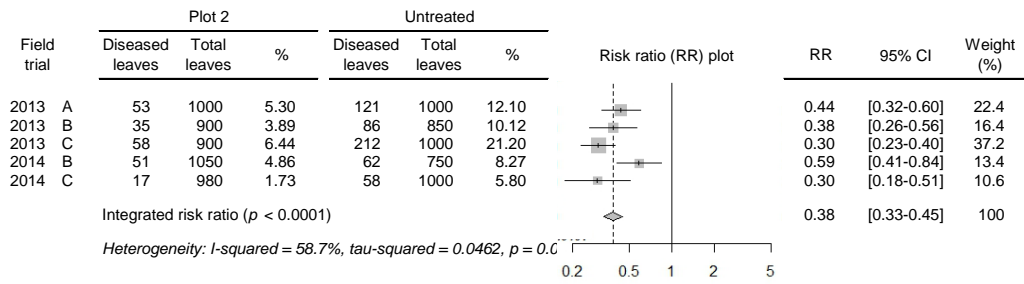
360

361

362



363



364

365

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

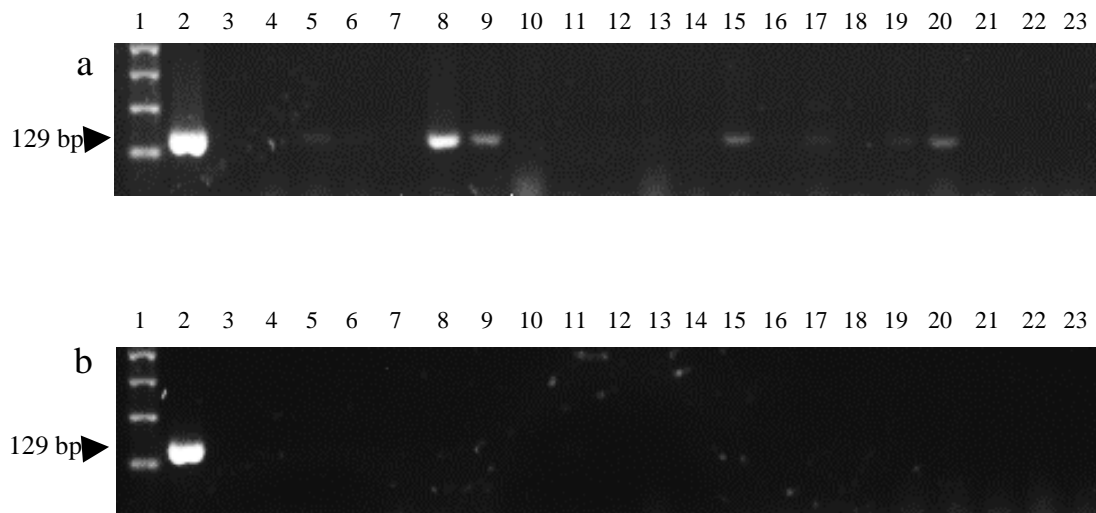
For each test, 3 buds were collected from each of 20 vines before sprouting (April–May 2012 and 2013) and tested separately; nt = not tested

366

367

368

369 Fig. 2



370