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Distribution of copper resistance gene variants of *Xanthomonas citri* subsp. *citri* and *Xanthomonas euvesicatoria* pv. *perforans*

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Abstract: In Taiwan, numerous crops are threatened by *Xanthomonas* diseases such as citrus bacterial canker caused by *X. citri* subsp. *citri* and tomato bacterial spot mainly caused by *X. euvesicatoria* pv. *perforans*. Foliar sprays of copper-based bactericides have been frequently used for control of plant bacterial diseases. However, in Taiwan not much attention was paid on copper-resistant (Cu^R) *Xanthomonas* spp. and their impact on disease control efficacy of copper-based bactericides. In this study, Cu^R *Xanthomonas* isolates were collected from citrus and tomato in Taiwan. Compared with the pronounced effect on the copper sensitive isolate, spraying of copper hydroxide at the recommended rate of 0.5 kg/ha could not protect tomato plants against bacterial spot caused by the Cu^R isolate. Phylogenetic analysis of concatenated copper resistance genes, *copL*, *copA*, and *copB*, indicate that the Taiwanese Cu^R isolates belong to the worldwide clade. In addition to the three previously reported variants of the *copB* gene, analysis of complete *copB* sequences from xanthomonads associated with citrus and solanaceous hosts revealed the other three variants of *copB* and their global distribution. Copper-resistant *Xanthomonas* isolates from Taiwan have the two unreported variants of *copB* genes which differ from the other three previously reported types in the sizes and structures. The information provided here reveals the necessity to develop and include alternative measures rather than relying on foliar sprays of copper bactericides for sustainable control of tomato bacterial spot in Taiwan.

Keywords: citrus; efficacy; polymorphism; tomato; xanthomonad

In Taiwan, various economically important crops suffer from *Xanthomonas* diseases such as Asiatic citrus canker caused by *X. citri* subsp. *citri* (Lin 2012; Huang & Ni 2017) and tomato bacterial spot caused by *Xanthomonas vesicatoria*, *X. euvesicatoria* pv. *euvesicatoria* and *X. euvesicatoria* pv. *perforans* (syn. *X. perforans*) (Leu et al. 2010; Constantin et al. 2016). Copper-based bactericides have been widely used for control of plant bacterial diseases in the world including Taiwan. However, frequent applications of copper-based bactericides induce evolution and development of bacterial strains either resistant or tolerant to copper (Hseu & Hsu 1991; Wu et al. 1995; Canteros et al. 2008; Behlau et al. 2012b,

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2013). The intensive use of copper-based bactericides also results in phytotoxicity, soil accumulation of copper, and negative effects on soil biota as reviewed by Lamichhane et al. (2018).

Copper-resistant (Cu^R) xanthomonads have been found around the world (Behlau et al. 2011, 2013). Large-sized plasmids carrying copper resistance genes (*cop* genes) are predominantly present in Cu^R xanthomonads (Wu et al. 1995; Behlau et al. 2011, 2012a). The *cop* genes in xanthomonads have been identified and organized in a cluster (Behlau et al. 2011). In Cu^R *Xanthomonas* strains, *copL*, *copA*, and *copB* genes rather than the other genes in the *cop* cluster mainly contribute to copper resistance (Behlau et al. 2011). In addition to plasmid-borne *cop* genes, a distinct copper resistance gene cluster was found in the chromosome of *X. campestris* pv. *vesicatoris* XvP26 (Basim et al. 2005).

Phylogenetic analysis of *copL*, *copA*, and *copB* genes indicated that *copL* and *copA* are the least and most conserved copper resistance genes, respectively (Behlau et al. 2013). According to the origins of isolation, Cu^R *Xanthomonas* strains associated with citrus and solanaceous hosts can be categorised into four clades such as Argentina, Virgin Island, Florida and a worldwide clade by phylogenetic analysis of concatenated *copLAB* genes (Behlau et al. 2013). Most Cu^R strains were grouped into the worldewide clade (Behlau et al. 2013). In addition, sequence alignment of complete *copB* sequences from Cu^R *Xanthomonas* strains revealed three variants of *copB* genes with different lengths and gaps. *X. euvesicatoria* 81–23, *X. citri* subsp. *citri* A44, and *Xanthomonas* sp. 1219., the three reference Cu^R strains, have the small-sized 1 158 bp, intermediate-sized 1 296 bp, and large-sized 1 425 bp *copB* genes, respectively (Behlau et al. 2013). Recently, a unique *cop* gene cluster was identified from the plasmid of *X. campestris* pv. *campestris* BrA1 (Behlau et al. 2017).

Occurrence of Cu^R isolates of bacterial spot pathogens in Taiwan were firstly reported in 1991 and have been found in major production regions of tomato and pepper up to date (Hseu & Hsu 1991; Wu et al. 1995; Basim et al. 1999; Burlakoti et al. 2018). However, not much attention was paid on the of Cu^R *Xathomonas* spp. Moreover, it was no evidence of the influence of Cu^R *Xathomonas* spp. on disease control efficacy of copper-based bactericides applied at the recommended rate in Taiwan. Thus, the aims of this study were to survey Cu^R *Xanthomonas* isolates in Taiwan, to evaluate the efficacy of copper bactericide on control of tomato bacterial spot caused by the isolates sensitive and resistant to copper, and to analyze sequences of copper resistance genes.

MATERIAL AND METHODS

Bacteria and culture media. In our collections, 34 and 28 isolates of *X. citri* subsp. *citri* and *X. euvesicatoria* pv. *perforans*, respectively, were collected from 2015 through to 2020 from the main locations of citrus and tomato production (Table 1). Isolates of *X. citri* subsp. *citri* and *X. euvesicatoria* pv. *perforans* populations, which were collected from the orchards that had never been sprayed with copper-based bactericides, were considered nonexposed populations. The rest were collected from orchards and nurseries where copper-based bactericides were used to control the disease. Unfortunately, records of bactericide applications were not available from all of these locations.

X. citri subsp. *citri* and *X. euvesicatoria* pv. *perforans* isolates used in this study were listed in Table 1.

Table 1. *Xanthomonas* isolates from various hosts and locations throughout Taiwan and the frequency of phenotypic responses observed with $CuSO_4$ at 0.8 mM

Location	Host	Year	No. of isolates	Exposed to copper	Copper sensitivity	
					sensitive	resistant
Chiayi	citrus	2015	30	no	30	0
Chiayi	tomato	2016	4	no	1	3
Shigang	citrus	2017	4	yes	2	2
Shueishang	tomato	2018	4	n.a.	0	4
Taipao	tomato	2018	10	yes	1	9
Liujiao	tomato	2018	10	yes	0	10
Chiayi	citrus	2020	4	no	3	1

n.a. – information not available

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All *Xanthomonas* isolates were kept at –80 °C in LB broth (Sambrook & Russel 2001) with 20% glycerol for long-term storage. Xanthomonads were routinely cultured on Nutrient agar (NA) (Difco™, USA) at 28 °C for 24 h for further assays.

Determination of copper resistance level of *Xanthomonas* isolates. Copper sensitivity test was performed according to the method of Behlau et al. (2013) with slight modifications. For consistency, NA was used in copper sensitivity test. *Xanthomonas* isolates, cultured overnight on NA plates, were streaked on NA plates including 0, 0.1, 0.2, 0.4, 0.6, 0.7, 0.8, 1.6, and 3.2 mM $CuSO_4$. Each assay was performed in triplicate. Inoculated plates were incubated at 28 °C for two days and bacterial growth was assessed. The level of copper resistance/tolerance was rated as described by Behlau et al. (2013) and Marin et al. (2019). Stains sensitive, tolerant, or resistant to copper were differentiated when they were able to grow on NA plates including ≦ 0.6, 0.6–0.8 and ≧ 0.8 mM $CuSO_4$.

Disease control assay. Tomato plants (cv. To-001) were grown according to Tsai et al. (2011). One-month old tomato plants were used for disease control experiments.

Copper hydroxide (water dispersible granules, 53.8% active ingredient, DuPont, Taiwan) was dissolved in distilled water to final concentrations of 0.5 g/L according to the governmental regulation of Taiwan and instructions. Tomato plants were sprayed with copper hydroxide solution using a handheld small sprayer at the rate of 0.5 kg/ha officially approved by the government in Taiwan. Distilled water was used as the control. One day post treatment, tomato leaves were misted with bacterial suspensions of the copper-sensitive (Cu^S) isolate XpT2 and the Cu^R isolate T0709-01 at 1×10^8 CFU/mL until running-off onto abaxial surfaces of leaves. The inoculated tomatoes were kept under moist condition at 25 °C for 3 days and then placed on the greenhouse bench. One week post inoculation, numbers of spots per leaf were measured. The disease severity of inoculated plants (ten leaves per plant) were visually assessed using a 0–4 scale: 0 = no symptoms; 1 = 1–3 spots; 2 = 4–6 spots; 3 = 7 or more spots but no coalescence of spots; 4 = 7 or more spots with coalescence of spots. This experiment had three replications (three plants per replicate).

Detection, sequencing, and phylogenetic analysis of copper resistance genes. Partial sequences of *copL*, *copA*, and *copB* genes from *Xanthomonas* isolates (Table 2) were amplified by PCR with the primer pairs specific to the three plasmid-borne copper resistance genes (Behlau et al. 2013). Complete sequences of the *copB* gene were amplified with the primers copBBF/copBBR (Behlau et al. 2013). Sequencing of the amplicons was conducted by Genomics (New Taipei City, Taiwan). Obtained sequences were submitted to the BLAST search engines of NCBI GenBank. The sequences of the representative isolates were deposited in GenBank (Table 3).

In addition to sequence comparison, phylogenetic analysis of individual and concatenated *copLAB* sequences were analyzed by using MEGA7 (Kumar et al. 2016). Alignments for the sequences including our sequence data and reference sequences were conducted using ClustalW and afterwards edited manually. The sequences of the reference strains used in the study by Behlau et al. (2013) and Richard et al. (2017) were retrived from GenBank. Maximum likelihood (ML) method was used for the phylogenetic analysis, and the bootstrap was performed with 1 000 replicates.

Statistic analysis. All data were statistically analyzed using the PAST3 software (version 2.17C) package for Windows (Hammer et al. 2001). Data were analyzed using nonparametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$).

RESULTS

Determination of copper resistance level of *Xanthomonas* isolates. In our collections, 2 and 26 of isolates of *X. citri* subsp. *citri* and *X. euvesicatoria* pv. *perforans*, respectively, were resistant to copper (Table 1). The tested *Xanthomonas* isolates with copper resistance were able to grow on NA plates supplemented with 0.8 mM $CuSO_4$, whereas the isolates T0319-02 and XpT40 exhibited confluent growth in the presence of 3.2 mM $CuSO_4$ (Table 2). The isolates without copper resistance/tolerance could not grow on NA plates supplemented with more than 0.4 mM $CuSO_4$.

All isolates of *X. citri* subsp. *citri* collected from the orchards that had never been sprayed with copper-based bactericides exhibited sensitivity to copper. However, most isolates of *X. euvesicatoria* pv. *perforans*, including exposed and nonexposed populations, exhibited copper resistance. Of 28 *X. euvesicatoria* pv. *perforans* isolates, only the isolates T0319-03 and XpT2 could grow on NA plates supplemented with as much as 0.4 mM

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Table 2. *Xanthomonas* isolates tested for copper resistance and the presence of copper resistance genes

Orgnism	Isolate	Origin	Year	Source	Co^R	Level of resistance (mM $CuSO_4 \cdot 5H_2O$)*	PCR product size (bp)**			
							copL	*copA*	*copB*	full *copB*
Xcc	T2	Taiwan	2017	citrus	+	+ 0.8	356	870	384	1 125
	T4	Taiwan	2017		+	+ 0.8	356	870	384	1 125
	5-2	Taiwan	2020		+	+ 0.8	356	870	384	1 125
Xep	T0709-01	Taiwan	2016	tomato	+	± 3.2	356	870	531	1 272
	T0709-02	Taiwan	2016		+	+ 0.8	356	870	531	1 272
	T0709-03	Taiwan	2016		–	+ 0.4	n/a	n/a	n/a	n/a
	T0709-04	Taiwan	2016		+	+ 0.8	356	870	531	1 272
	T0319-01	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	T0319-02	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	T0319-03	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	T0319-04	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT2	Taiwan	2018		–	+ 0.4	n/a	n/a	n/a	n/a
	XpT3	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT4	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT5	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT9	Taiwan	2018		+	+ 0.8	356	870	531	1272
	XpT11	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT12	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT13	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT14	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT16	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT31	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT32	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT33	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT34	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT35	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT36	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT37	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT38	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT39	Taiwan	2018		+	+ 0.8	356	870	531	1 272
	XpT40	Taiwan	2018		+	± 3.2	356	870	531	1 272

*highest concentration of $CuSO_4 \cdot 5H_2O$ supplemented into NA plates with confluent (+) or weak (±) bacterial growth; **the copper resistance genes were amplified with the primers described by Behlau et al. (2013); Xcc – *Xanthomonas citri* subsp. *citri*; Xep – *Xanthomonas euvesicatoria* pv. *perforans*; Co^R – the copper resistance; n/a – not applicable because no PCR products were obtained

$CuSO_4$, indicating both isolates exhibited sensitivity but not tolerance to copper.

Effect of copper bactericide on controlling tomato bacterial spot. Since fungicide application is strictly regulated in Taiwan, the effect of copper hydroxide at the officially approved rate alone on suppression of tomato bacterial spot was evaluated. While applied at the recommended rate one day before inoculation, a foliar spray of copper hydroxide could significantly but not completely suppress the severity of tomato bacterial spot caused by the Cu^S isolate XpT2 in comparison with the control treatment (Figure 1). In contrast, the severity of tomato bacterial spot caused by the Cu^R isolate T0709-01 was similar in the control and copper treatment (Figure 1).

Comparison and phylogenetic analysis of partial sequences of copper resistance genes. The copper resistance genes, *copL*, *copA*, and *copB*, of all Cu^R isolates were successfully amplified by PCR. The sizes of partial *copL* and *copA* sequences from our *Xanthomonas* isolates are identical (Table 2). The partial

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Table 3. Global distribution of six *copB* variants from *Xanthomonas* strains associated with citrus and solanaceous hosts

Species	Strain	Year	Origin	Host	*copB* variant	Reference
Xcc	A44	2003	Argentina	citrus	I	Behlau et al. 2013
Xsp	1219	1995	Ohio, USA	n.a.	II	Behlau et al.2013
Xe	81-23	1981	Florida, USA	pepper	III	Behlau et al. 2013
Xcc	T4	2017	Taiwan	citrus	IV	this study
Xep	T0709-01	2016	Taiwan	tomato	V	this study
Xe	LMG 930	1996	America	pepper	VI	Richard et al. 2017
Xv	LMG 911	1955	New Zealand	tomato	V	Richard et al. 2017
Xep	LH3	2010	Mauritius	tomato	IV	Richard et al. 2017
Xep	LM159	1987	Argentina	pepper	V	Richard et al. 2017
Xhg	ICMP 7383	1980	New Zealand	tomato	I	Richard et al. 2017
Xcc	LH201	2010	Reunion	citrus	V	Richard et al. 2017
Xcc	LH276	2010	Reunion	citrus	V	Richard et al. 2017
Xcc	LJ207-7	2012	Reunion	citrus	I	Richard et al. 2017
Xcc	LL074-4	2014	Martinique	citrus	I	Richard et al. 2017
Xhg	JS749-3	1997	Reunion	tomato	I	Richard et al. 2017

Xcc – *Xanthomonas citri* subsp. *citri*; Xsp – *Xanthomonas* sp.; Xe – *Xanthomonas euvesicatoria*; Xep – *Xanthomonas euvesicatoria* pv. *perforans*; Xhg – *Xanthomonas hortorum* pv. *gardneri*; n.a. – information not available

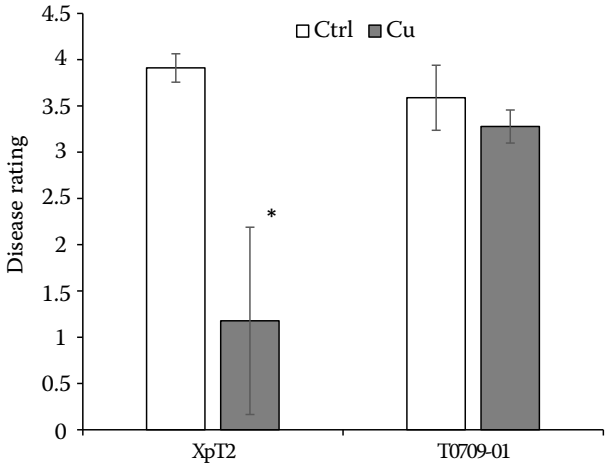

Figure 1. Effect of copper hydroxide on bacterial spot disease caused by copper-sensitive and copper-resistant *Xanthomonas euvesicatoria* pv. *perforans*

Disease severity of bacterial spot on tomato leaves with a foliar spray of copper hydroxide (Cu) or not (Ctrl); XpT2 – the copper-sensitive isolate; T0709-01 – the copper-resistant isolate; XpT2 and T0709-01 used as the inocula; bars indicated with the asterisk are statistically different based on nonparametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$)

copB sequences can be categorized into two groups based on their sizes as described by Behlau et al (2013). The *copB* sequences of each group have the same size and originate from the same host species (Table 2).

The partial sequences of *copL*, *copA*, and *copB* genes were compared by blastn analysis against the sequences in GenBank. The *copL* and *copA* sequences of all sequenced isolates were 99.5–100% and 100%, respectively, identical with those of *Xanthomonas* spp. A blastn search showed the *copB* sequences of all sequenced isolates sharing 97.4% identity with those of *Xanthomonas* spp. Moreover, the *copA* and *copB* sequences of our isolates shared only 78% and 72% identity, respectively, to those of *X. campestris* pv. *campestris* BrA1 (Behlau et al. 2017). No significant similarity was found between the *copAB* sequences of our isolates and the chromosomal *copAB* of XvP26 (Basim et al. 2005).

According to the ML phylogeny calculated from the concatenated *copLAB* alignment (Figure 2), the topology of the constructed phylogenetic tree resembled that reported in the study of Behlau et al. (2013). All our isolates clustered in the worldwide clade. More specifically, the isolates of *X. citri* subsp. *citri* clustered together with *X. euvesicatoria* strains Xv669 and Xv818 isolated in Puerto Rico and Spain, respectively. Our isolates of *X. euvesicatoria* pv. *perforans* formed a homogenous group and constituted a sister clade to *Xanthomonas* sp. 1219, a pathogenic strain originated from Ohio.

Sequence analysis of complete *copB* genes from Cu^R xanthomonads. Analysis of complete

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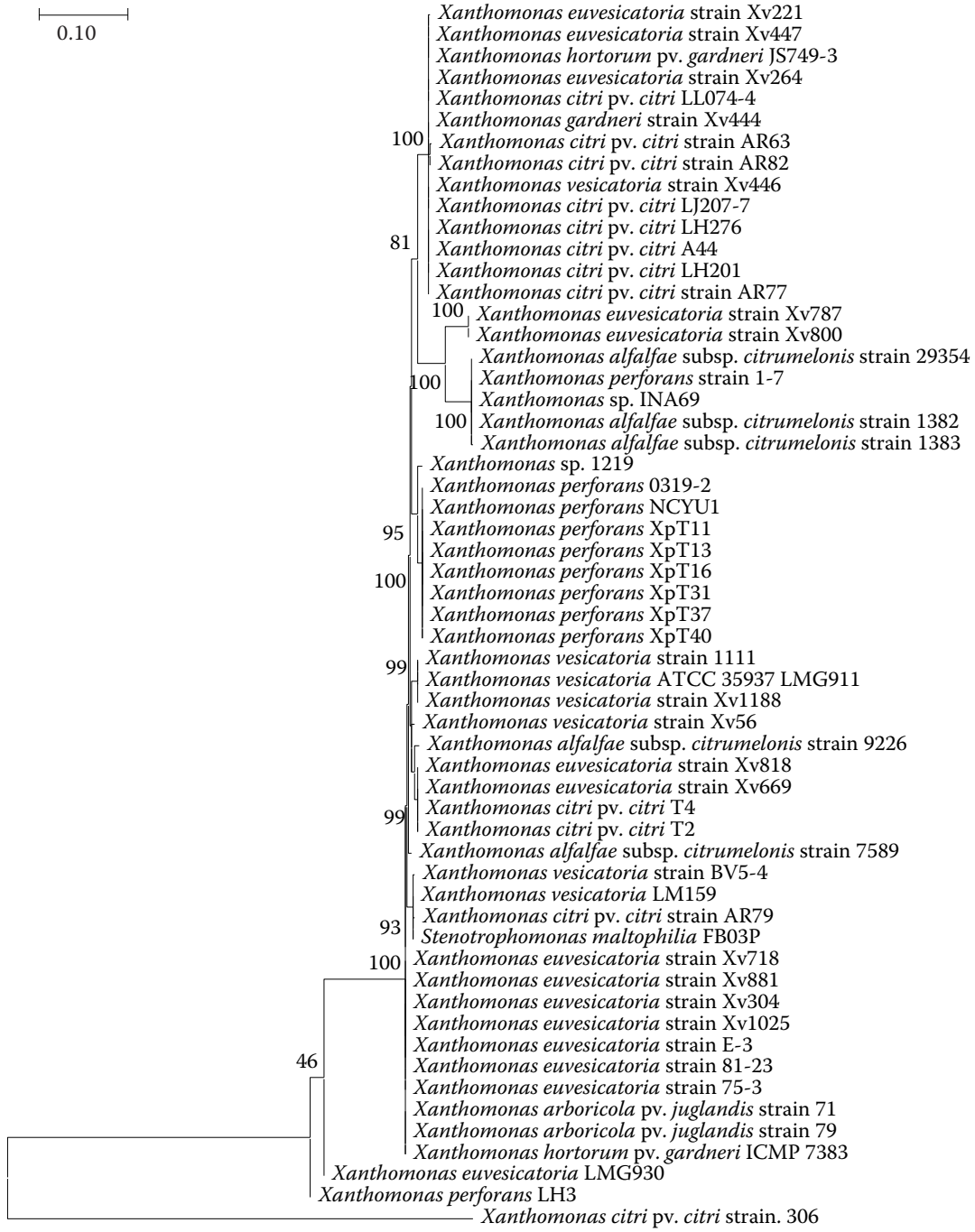

Figure 2. Maximum-likelihood phylogeny based on three concatenated partial sequences of *copL*, *copA*, and *copB* genes from copper-resistant *Xanthomonas* spp.

The concatenated *cohL*, *cohA*, and *cohB* sequences from *Xanthomonas citri* subsp. *citri* A306 were used as the outgroup; bootstrap values based on 1 000 replicates are shown at branch nodes

copB sequences from the Taiwanese isolates showed that 1 125 and 1 272 bp of *copB* exist in *X. citri* subsp. *citri* and *X. euvesicatoria* pv. *perforans*, respectively. The complete *copB* sequences of our isolates differ from those previously reported in the sizes and structures. The *copB* sequences of our *X. citri* subsp. *citri* and *X. euvesicatoria* pv. *perforans* isolates are shorter than those of *X. euvesicatoria* 81–23 (1 158 bp, the small-sized) and *X. citri* subsp. *citri* A44 (1 296 bp, the intermediate-sized), respectively.

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No *copB* sequences of our isolates were found similar to the large-sized 1 425 bp *copB* of *Xanthomonas* sp. 1219.

Moreover, the alignment of complete *copB* sequences of our *Xanthomonas* isolates with those of *X. citri* subsp. *citri* A44, *X. euvesicatoria* 81–23, *Xanthomonas* sp. 1219 and the other sequenced Cu^R xanthomonads revealed six variants of *copB* genes. We assign the *copB* sequences from *X. citri* sub sp. *citri* A44, *X. euvesicatoria* 81–23, *Xanthomonas* sp. 1219, *X. citri* subsp. *citri* T4, *X. euvesicatoria* pv. *perforans* T0709-01 and *X. euvesicatoria* LMG 930 to represent the variant I to the variant VI (Figure 3). Two new combinations of gaps in each *copB* sequence were observed from the Taiwanese isolates and one new combination from *X. euvesicatoria* LMG 930 (Figure 3). A combination of 300 and 36 bp gaps was found in the *copB* sequences of the collected *X. citri* subsp. *citri* isolates (Variant IV) and a combination of 153 and 36 bp gaps in the *copB* sequences of the collected *X. euvesicatoria* pv. *perforans* isolates (Variant V, Figure 3). The shortest 1 122 bp *copB* of *X. euvesicatoria* LMG 930 has a combination of 3, 300 and 36 bp gaps (Variant VI, Figure 3). The gaps locate in the same regions of *copB* genes as described in *X. citri* subsp. *citri* A44, *X. euvesicatoria* 81-23, and *Xanthomonas* sp. 1219 (Behlau et al. 2013). Like *Xanthomonas* sp. 1219, the *copB* of our isolates all have three bp encoding an extra codon at the *N*-terminal coding region (Figure 3).

Global distribution of *copB* genes. It was intriguing to understand the origin and global distribution of the six *copB* variants since the spread of *copB* genes was not clear. The origins and *copB* variants of 13 completely sequenced Cu^R xanthomonads and Taiwanese Cu^R isolates are presented in Figure 4 and Table 3. Variant I has been found in five strains from Argentina (2003, citrus), Martinique (2014, citrus), New Zealand (1980, tomato) and Reunion Island (1997, citrus and tomato). One strain with Variant-II *copB* and one with Variant III were both from the USA (1995 and 1981, respectively). Variant IV was found in one strain from Mauritius (2016, tomato) and in Taiwanese isolates (2017, citrus). Variant V has occurred in four strains from Argentina (1987, pepper), New Zealand (1955, tomato), and Reunion Island (2010, citrus) and in Taiwanese isolates (2016, tomato). Variant VI was only found in *X. euvesicatoria* LMG 930 from the USA (1996, pepper).

DISCUSSION

The Cu^R isolates of *X. citri* subsp. *citri* were found in citrus orchards exposed to copper bactericides and the nonexposed populations maintained their sensitivity to copper. Our data corroborates previous studies (Behlau et al. 2012a, b) indicating a risk for development of copper resistance in *X. citri* subsp. *citri* while cultivation of citrus under frequent exposure to copper bactericides. Moreover, we found that more than 90% of *X. euvesicatoria* pv. *perforans*

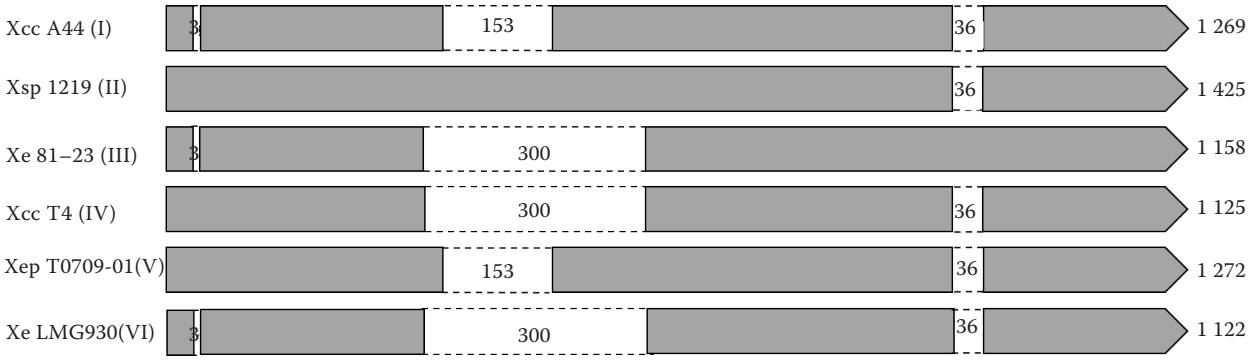

Figure 3. Schematic representation of six variants of *copB* gene from *Xanthomonas* spp. associated with citrus and solanaceous hosts

The complete *copB* sequences from two representative isolates, *X. citri* subsp. *citri* T4 (Xcc T4, Variant IV) and *X. euvesicatoria* pv. *perforans* T0709-01 (Xep T0709-01, Variant V), from Taiwan are presented; the complete *copB* sequences from three reference strains, *X. citri* subsp. *citri* A44 (Xcc A44, Variant I), *X. euvesicatoria* 81–23 (Xe 81–23, Variant II), and *Xanthomonas* sp. 1219 (Xsp 1219, Variant III) (Behlau et al. 2013), and one one sequenced strain *X. euvesicatoria* LMG 930 (Xe LMG 930, Variant VI) are included; Dashed shapes indicate sequence gaps compared with the other strains; the numbers at right and within the shapes indicate total sequence length and gap sizes in base pairs, respectively

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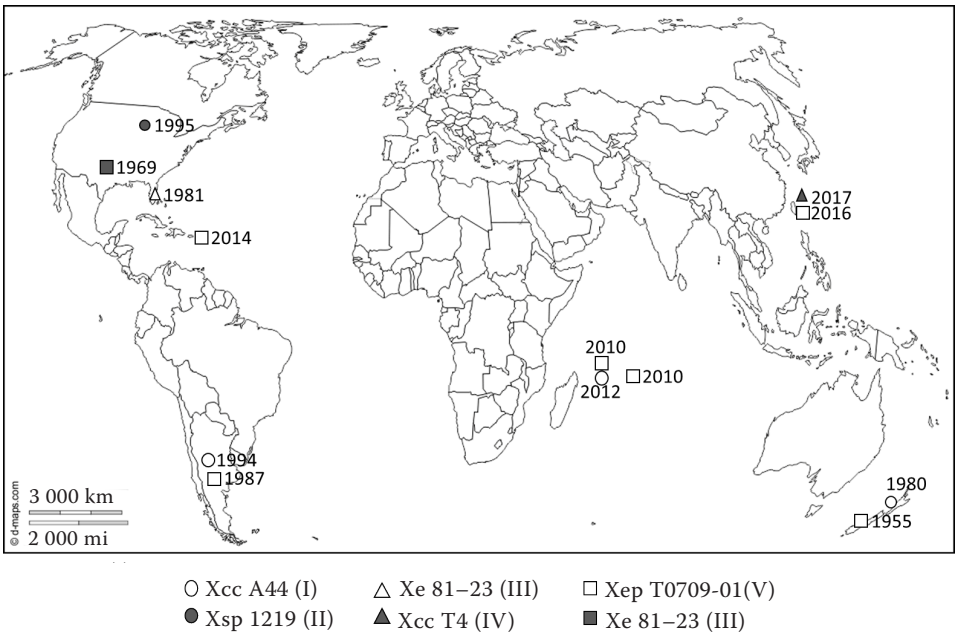

Figure 4. Global distribution of six variants of *copB* gene from *Xanthomonas* spp. associated with citrus and solanaceous hosts

The presence of the *copB* variants and the year of isolation are indicated

isolates collected in 2016–2018 are resistant to copper. Our data is in full agreement with the study from Burlakoti et al. (2018) which reported a shift of Cu^R populations of *X. perforans* from 2000 to 2016 in Taiwan. Surprisingly, Cu^R populations of *X. euvesicatoria* pv. *perforans* prevalently existed in the non-exposed nurseries of tomato. General occurrence of Cu^R populations of *X. euvesicatoria* pv. *perforans* is likely to be an emerging problem in Taiwan.

Copper resistance in plant pathogenic bacteria has been shown to contribute to a failure or significantly decreased efficacy or unstable controlling plant bacterial diseases by copper bactericides (Pernezny et al. 2008; Griffin et al. 2017; Zhang et al. 2017). Since various application programs were conducted by studies to evaluate the efficacy of copper-based bactericides (Griffin et al. 2017), we decided to fairly test the effect of copper hydroxide, which was applied at the officially approved rate one day before inoculation, on tomato against bacterial spot caused by the Cu^S and Cu^R isolates of *X. euvesicatoria* pv. *perforans*. A foliar spray of copper hydroxide one day before inoculation was not able to inhibit disease severity caused by the Cu^R isolate. Our data is in full agreement with the study of Zhang et al. (2017) which indicated the populations of Cu^R *P. syringae* pv. *phaseolicola* were not affected by sprays of copper hydroxide. In contrast, application of copper hydroxide in the same way significantly inhibited both disease severity caused by the Cu^S pathogen and bacterial growth *in planta*. Accordingly, a foliar spray of copper bactericide can provide pronounced protection against the Cu^S pathogen but not the Cu^R pathogen. Our data implies that the copper resistance in *X. euvesicatoria* pv. *perforans* contributed to the failure of disease control in practice with copper bactericides at the rate approved by the Taiwan government. Since Cu^R populations of *X. euvesicatoria* pv. *perforans* prevalently existed in the exposed and non-exposed nurseries of tomato, it is necessary to develop and include alternative measures for effective sustainable control of tomato bacterial spot in Taiwan.

Furthermore, our data indicated that the rate (0.5 kg/ha) of copper hydroxide approved by the Taiwan government could not sufficiently control tomato bacterial spot caused by Cu^R isolates. Either a higher dose (3 g/L) of copper hydroxide or a mixture of copper hydroxide and mancozeb have proved to provide significantly better efficacy against tomato bacterial spot in comparision with application of copper hydroxide at the lower rate alone (Stall et al. 1980; Canteros et al. 2017). In addition to controlling bacterial spot, mixtures of copper and mancozeb were compatible for control of other fungal/oomycete pathogens in tomato such as *Phytophthora infestans* and *Stemphylium*

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solani (Conover & Gerhold 1981). Accordingly, we suggest the government to reconsider, evaluate and approve a sufficient measure for copper bactericide to control tomato bacterial spot and other *Xanthomonas* plant diseases in practice.

Global trade of *Xanthomonas*-contaminated tomato seeds was suggested to be associated with the global spread of bacterial spot pathogens (Potnis et al. 2015). A low incidence of seed transmission was speculated able to introduce exotic *Xanthomonas* strains (Pohronezny et al. 1992). Given that hybrid tomato seeds are mainly produced outside of Taiwan (Burlakoti et al. 2018), Cu^R isolates of *X. euvesicatoria* pv. *perforans* could be originally imported to Taiwan by global trade of tomato seeds. Phylogenetic analysis of the combined sequences of *copLAB* revealed that the Cu^R isolates of two *Xanthomonas* species collected in Taiwan belong to the worldwide clade (Figure 2). The data is in full agreement with the study of Behlau et al. (2013), supporting the occurrence of independent exchange of *cop* genes among different *Xanthomonas* species.

Given that the *copLAB* clusters are all located in the plasmids (Behlau et al. 2013, 2017; Richard et al. 2017), we assume the *cop* genes of the Taiwanese isolates could be carried by Cu^R plasmids. Moreover, copper resistance is more frequently transferred between bacteria when the Cu^R genes are located in the plasmids (Basim et al. 1999; Behlau et al. 2012a). Further studies with whole genome sequencing will clarify if the *cop* genes of our isolates are borne in Cu^R plasmids (Richard et al. 2017).

It was intriguing to understand if interspecies exchange of *cop* genes between *X. citri* subsp. *citri* and *X. euvesicatoria* pv. *perforans* in Taiwan. Hence, the complete *copB* sequences of xanthomonads associated with citrus and solanaceous hosts were analyzed. We discovered three previously unreported variants of *copB* genes. That also suggests possible evidence of independent exchange of the *cop* genes among xanthomonads in Taiwan. Variant IV, which was found in our Cu^R *X. citri* subsp. *citri* isolates, is also carried in *X. euvesicatoria* pv. *perforans* LH3 from Mauritius in 2010 (Table 3). Variant V, which was found in our Cu^R *X. euvesicatoria* pv. *perforans* isolates, could have originated from *X. vesicatoria* LMG 911 from New Zealand in 1955 and subsequently spread to Argentina and Reunion Island (Figure 3). Furthermore, the host range of the Cu^R plasmid carrying Variant V of the *copB* gene was expanded to *X. citri* subsp. *citri*. Accordingly, we suggest that Variants IV and V of the *copB* gene in our isolates could be received from Cu^R xanthomonads in other regions but not Taiwan.

The Cu^R xanthomonads carrying Variants I and V of the *copB* gene were firstly isolated in New Zealand in 1980 and 1955, respectively, suggesting that New Zealand could be the origin of these two variants. Both Variants I and V of the *copB* gene have been widely distributed in Central and South America, the islands in the Indian Ocean and the southwestern and western Pacific Ocean (Figure 4). Variant IV has been found in the islands in the Indian Ocean and western Pacific Ocean (Figure 4). In addition to geographic distribution, these three variants are carried by the xanthomonads pathogenic to citrus and tomato/pepper, suggesting these variants could originate from various *Xanthomonas* spp. or are horizontally transferred between *Xanthomonas* spp. associated with citrus and solanaceous hosts in the same geographic region, for instance, Reunion Island.

Unlike what occurred in Reunion Island, the concatenated ML phylogeny implies that the partial sequences of *Xanthomonas cop* genes from Taiwan are highly related to their bacterial hosts. Moreover, Variants IV and V of the *copB* sequences were only found in our Cu^R *X. citri* subsp. *citri* and *X. euvesicatoria* pv. *perforans* isolates, respectively. We hypothesize that, in addition to independent genetic exchange, *cop* genes could co-evolve with the bacterial hosts in Taiwan. It will be intriguing to continuously survey and analyze the *copLAB* and whole genome sequences from Cu^R xanthomonads in Taiwan in subsequent studies.

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