

# Bacterial community associated with canker disease from sweet cherry orchards of central valley of Chile presents high resistance to copper

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Received: 2 February 2021; Accepted: 3 May 2021; doi:10.4067/S0718-58392021000300378

## ABSTRACT

The Chilean sweet cherry (*Prunus avium* (L.) L.) industry became the leading exporter worldwide. The bacterial canker is the most significant disease causing major economic losses. *Pseudomonas syringae* pv. *syringae* (Pss) is the only related pathovar recognized in Chile and it is mainly controlled with Cu-based antimicrobial compounds (CBAC). Soil contamination and the decreasing efficacy of CBACs by the emergence of Cu-resistant bacterial strains threaten the long-term sustainability of sweet cherry production. This study aimed at characterizing the bacterial community associated with canker infection injuries by assessing Cu resistance in sweet cherry orchards in the O'Higgins Region of Chile. Eighty bacteria isolates were obtained from tissue that presented bacterial canker symptoms from cherry trees. We assessed the production of fluorescent pigments in Fe-deficient media, presence of the housekeeping genes *rpoD* and *cts*, and presence of syringomycin-producing genes *syrb* and *syrd*. Their pathogenic ability was evaluated on immature sweet cherry fruits and Cu resistance was determined as the minimum inhibitory concentration (MIC) using CuSO<sub>4</sub>. Only five isolates were Cu-susceptible (MIC < 0.8 mM), while 75 isolates exhibited different levels of Cu resistance (MIC > 0.8 mM). At least one gene of the *copABCD* operon/or and its regulatory genes were detected in 17 isolates, suggesting that most isolates likely have different mechanisms of Cu tolerance. Six isolates were identified as Pss, presenting different degrees of Cu resistance, but all presented at least one feature of the *copABCD* operon. A bacterial community that presents high Cu-resistance, probably under control of diverse genetic mechanisms, decreases the efficacy of CBAC.

**Key words:** Copper resistance, *Pseudomonas syringae* pv. *syringae* identification, sweet cherry.

## INTRODUCTION

The Chilean sweet cherry (*Prunus avium* (L.) L.) industry has grown notoriously in the last 15 yr. Between the period 2003-2018, the area under sweet cherry cultivation increased from 8531 to 34567 ha (ODEPA-CIREN, 2019). Most sweet cherry orchards and over 90% of production are located in central Chile, with 46.0% and 35.7% of this cultivated area in the Maule and Libertador General Bernardo O'Higgins Regions, with 17656 and 13699 ha, respectively. However, production in southern regions of Chile has increased significantly in recent years (ODEPA-CIREN, 2019). With per annum output of 233929 t, Chile is the 3<sup>rd</sup> largest producer of sweet cherries in the world, after Turkey and USA (FAOSTAT, 2021). However, Chile is the leading exporter worldwide, with more 146 kt, distantly followed by USA (87 kt), Turkey (60 kt), Uzbekistan (36 kt) and Spain (31 kt). Together these main exporters of sweet cherry constitute 64% of total exports (IndexBox, 2019). Chilean sweet cherries are exported to more than 50 countries, but 87.4% of production goes to the Chinese market (Chile-Customs, 2019).

Bacterial canker is a problem for the sweet cherry industry globally (Spotts et al., 2010). Typical symptoms include blossom blast, twig blight, shoot dieback, leaf shot holes and trunk and branch cankers with associated gummosis. When the outer tissue of the canker area is cut away, the tissue underneath typically exhibits reddish-brown discoloration (Kennelly et al., 2007) that may also take the form of vertical streaks associated with vascular tissue. Three pathovars of *Pseudomonas syringae* have been identified as causal agents of sweet cherry bacterial canker, *P. syringae* pv. *syringae* (Pss), *P. syringae* pv. *morsprunorum* race 1 (Psm R1) and *P. syringae* pv. *morsprunorum* race 2 (Psm R2) (Bultreys and Kaluzna, 2010). Of the three pathovars, only Pss has been identified in Chile from reports in the 1970s (Latorre and Waissbluth, 1979) and since then there have no subsequent reports. Recently a preliminary report identified Psm R1 in southern Chile, but it still under official quarantine. These epiphytic bacteria grow on the surfaces of aerial parts of cherry trees and weeds. The infection occurs in dormant woody tissue through leaf scars and wounds, colonizing cambial tissue and causing cankers in spring (Kennelly et al., 2007). The pathogen has the ability to kill both young and old trees, and is particularly devastating in young orchards. It was reported to have caused up to 75% loss of trees in Oregon, USA (Spotts et al., 2010). Economic losses in cherry trees caused by bacterial canker can reach 75% the first years after planting, and between 10% and 20% among adult trees (Spotts et al., 2010).

Chemical control of this disease is mainly limited to Cu-based antimicrobial compounds (CBAC) (Kennelly et al., 2007). Among the advantages of CBAC is their relatively high toxicity to plant pathogens, low cost, low toxicity to mammals, chemical stability, which prevents them from being readily washed from plant surfaces, and long residual periods (Cha and Cooksey, 1991). However, in recent years, growers have noted a reduction in the efficacy of the treatments which, in turn, has led to an increase in the dosage and frequency of Cu applications (Lamichhane et al., 2018). The excessive use of CBAC has resulted in the emergence of Cu-resistant bacterial strains, which raises concerns about the long-term sustainability of agricultural production (Lamichhane et al., 2018).

Copper resistance is a widespread phenomenon in *P. syringae*, which are pathogens of many hosts in which Cu is used to control disease (Sundin and Bender, 1993; Ladomersky and Petris, 2015). Copper resistance has been detected in Pss strains from cherry in Michigan (Sundin, 1989) and Turkey (Husseini and Akköprü, 2020), from mango in Spain (Kennelly et al., 2007) and strains of *P. syringae* associated with kiwi production (Colombi et al., 2017). The molecular basis of resistance to Cu in bacteria has been extensively studied since the late 1980s. Copper tolerance in bacteria occurs via three main mechanisms: (i) transmembrane export from the cytoplasm to periplasmic or extracellular space; (ii) sequestration by metallothioneins; and (iii) oxidation of Cu<sup>+</sup> by multi-copper oxidases to generate the less toxic Cu<sup>+2</sup> ion (Ladomersky and Petris, 2015).

Cellular Cu sequestration has been suggested as the main mechanism of Cu resistance in strains of *P. syringae*, explained by the presence of plasmid pPT23D (Cooksey, 1994). When bacteria grow on Cu-amended media, strains harboring plasmid pPT23D accumulate Cu, indicating that resistance is due to an uptake and sequestration mechanism (Cooksey, 1994). The plasmid pPT23D contains the Cu-resistant operon *copABCD* that encodes four proteins, CopA, B, C, and D, which is controlled by a Cu-inducible promoter and the regulatory genes *copR* and *copS* that encode a Cu-responsive signal transduction system (Cha and Cooksey, 1991). This operon confers Cu resistance to *P. syringae* at least in part by sequestering and accumulating Cu in the periplasm with Cu binding proteins, which may prevent toxic levels of Cu from entering the cytoplasm (Cha and Cooksey, 1991).

Although the first report of the bacterial canker disease in sweet cherry was in the 1970s (Latorre and Waissbluth, 1979; Latorre et al., 1985), the causal agent has not yet been well-characterized. Pss was identified as the causal agent of bacterial canker disease at a time when only 1700 ha in Chile were devoted to growing sweet cherries. Presently, with more than 34567 ha under cultivation, the conditions for the sweet cherry industry are very different in term of area, varieties, rootstocks and agronomic management. This study is aimed at characterizing the bacterial community associated with canker infection injuries by assessing Cu resistance in sweet cherry orchards in the O'Higgins Region one of the main sweet cherry producer in Chile.

## MATERIALS AND METHODS

### Sampling and bacteria isolation

Bacterial samples were collected in 2017 and 2018 from 18 commercial sweet cherry (*Prunus avium* (L.) L.) orchards in 11 cities, which are distributed along the central valley of the Libertador General Bernardo O'Higgins Region, Chile.

An area that is representative of the sweet cherry production in a Mediterranean climate. Trees were screened at shoot emergence and the beginning of bloom to identify individuals that presented symptoms of bacterial canker infection. Two types of samples were collected, one was tissue from the edge of cankers and the other were buds and leaves from symptomatic trees. Both sample types were bagged, properly labeled and sent in a cooler to the laboratory for bacterial isolation. The samples were carefully excised in the laboratory to avoid cross-contamination. Under a laminar flow cabinet, tissues from canker edges were disinfected by dipping them in 70% ethanol for 2 min and then in 1% NaOCl for 1 min, rinsed three times with sterile distilled water and finally dried with sterile absorbent paper. The outer bark was removed with a sterile instrument and the tissue cut into small pieces that were plated on *Pseudomonas* Agar F (Difco, Franklin Lakes, New Jersey, USA). Buds and leaves were crushed in a sterile mortar and placed in a glass flask with 50 mL distilled sterile water, and then shaken at 120 rpm (orbital benchtop shaking incubator ZWY-103D, Labwit Scientific, Burwood East, Victoria, Australia) for 1 h at room temperature. The bacterial suspension was serially diluted sufficiently to allow discrete bacterial colonies to form. The dilutions were sowed on plates with *Pseudomonas* Agar F amended with 100 µg mL<sup>-1</sup> cycloheximide (C7698-5G, Sigma-Aldrich, St. Louis, Missouri, USA). Plates from both types of samples were incubated in a growth chamber at 26 °C for 2-5 d. Fluorescent colonies were visualized under UV light (benchtop UV transilluminator, Analytik Jena US LLC, Upland, California, USA). A pure culture of a representative colony was taken from the plates and cultured in 5 mL King's medium B (King et al., 1954) until 0.4 OD, and then prepared as 70% glycerol stock culture that was aliquoted and stored at -80 °C.

### Genomic DNA isolation and molecular test

Broth cultures were harvested at the end of the exponential growth phase (0.5 OD). A small cell pellet was obtained and centrifuged at 6000 × g for 15 min. The DNA was extracted using the Qiagen DNeasy Ultra Clean Microbial kit (QIAGEN, Germantown, Maryland, USA) following the manufacturer's protocols. DNA quality and quantity were checked by agarose gel electrophoresis and spectrophotometric measurement of UV absorption at 260 and 280 nm and its ratio of 260/280, using an Infinite M200Pro Nanoquant (Tecan Group US, Morrisville, North Carolina, USA). A PCR assay that amplified a region of the 16S rRNA (Turner et al., 1999) was used as an indicator of good quality (positive control test). Then PCR assays were conducted with every bacterial isolate to identify the *Pseudomonas syringae* group and its pathovar Pss, using specific primers to encode sigma factor 70 (*rpoD*) and citrate synthase (*cts*) (Sawada et al., 1999; Sarkar and Guttman, 2004). The capacity to produce the toxin syringomycin specific to Pss was tested by detecting two genes responsible for its production, *syrB*, with a 752-bp product, and *syrD*, with a 446-bp product (Sorensen et al., 1998). Amplifications were conducted according to the condition described by the respective authors mentioned above. The DNA of the Pss strain (DSM Nr: 10604) obtained from the Leibniz Institute, DSM-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) was used as a positive control. The main primer features used in this study are described in Table 1. PCR products were analyzed by electrophoresis in 1.5% agarose gel according to Sarkar and Guttman (2004). Bacterial isolates that showed molecular profile of Pss were confirmed by LOPAT tests (L for levan production, O for oxidase production, P for pectinolytic activity, A for arginine dihydrolase production and T for tobacco sensitivity), and ice nucleation activity according to Lelliott et al. (1966).

**Table 1. PCR primers used in this study to detect different sets of genes in bacterial isolates.**

Gene	Primer sequence (5'--> 3')		T <sub>m</sub> (°C)	Expected size (bp)	Reference
	Forward	Reverse			
16s (27F-1492R)	AGAGTTTGATCMTGGCTCAG	GGTACCTTGTACGACTT	60	1465	Turner et al. (1999)
<i>rpoD</i>	AAGGCGARATCGAAATCGCCAAGCG	GGAACWKGCGCAGGAAGTCGGCAGC	63	580	Sawada et al. (1999)
<i>cts</i>	AGTTGATCATCGAGGGCGCWGCC	TGATCGGTTTGTATCTCGCACGG	56	610	Sarkar and Guttman (2004)
<i>syrB</i>	CTTCCGTGGTCTTGATGAGG	TCGATTTTGGCCGTGATGAGTC	62	752	Sorensen et al. (1998)
<i>syrD</i>	AAACCAAGCAAGAGAAGAAGG	GGCAATACCGAACAGGAACAC	62	446	Sorensen et al. (1998)
<i>copA</i> _P <sub>syr</sub> _1493	ACTCACCCAATCCATCTGCA	GAACCTCGCGGAACATACCC	59	196	Petriccione et al. (2017)
<i>copB</i> _P <sub>syr</sub> _1494	GCCTGTCTGAAACCGAAGTC	ATACGCACACCCAGGACTAG	59	166	Petriccione et al. (2017)
<i>copC</i> _P <sub>syr</sub> _1495	CCAAGCTGGTFTTCTTCGACT	GCTGTTTTGACTCCCATCGG	59	163	Petriccione et al. (2017)
<i>copD</i> _P <sub>syr</sub> _1496	TGCGGACATCCTTCATCTGT	CTATTAGCGCCACGATCACC	58	167	Petriccione et al. (2017)
<i>copR</i> _P <sub>syr</sub> _1497	GGTACTGTTTCTGACCGCAC	AAGGTCTCCGATTGCAAGC	59	181	Petriccione et al. (2017)
<i>copS</i> _P <sub>syr</sub> _1498	TTGCAGAACCTCCCAATCA	AATAGAACGCCCCAGAACCA	59	220	Petriccione et al. (2017)

### Pathogenicity test

The pathogenicity test was performed on immature cherries using the method described by Bultreys and Kaluzna (2010). Freshly collected immature sweet cherries at the straw yellow stage were disinfected by dipping them in 50% ethanol for 3 min, then rinsing them three times with sterile distilled water, and blotting them dry with tissue paper. The cherries were inoculated by pricking each in two places (2 mm deep) with a sterile needle with a suspension of a bacterial isolate (culture grown in King's medium B at 0.4 OD at 600 nm). Every bacterial isolate was inoculated in 10 cherries. After inoculation, the cherries were placed in transparent boxes lined with moist tissue paper to maintain humidity, and incubated at 22 °C with natural light (16:8 h photoperiod) for 10 d. The culture medium was inoculated as a negative control. Symptoms in terms of wounds around the inoculation sites were observed at days 3, 7, and 10 d post-inoculation (DPI). The average wound diameters of 10 cherries times two inoculation sites were registered at 10 DPI for all bacterial isolates. A mean wound diameter of over 2.0 mm was the criterion to consider an isolate as pathogenic. Statistical analyses were performed with R software 3.4.4v (R-Core-Team, Foundation for Statistical Computing, Vienna, Austria). A one-way ANOVA and the post-hoc Tukey HSD test were used to compare the pathogenicity of *P. syringae* isolates. GraphPad Prism version 6.00 (GraphPad Software, San Diego, California, USA) was used to make graphs.

### In vitro Cu-resistance and copABCD gene cluster detection

Resistance to Cu was assessed according to Cazorla et al. (2002) to determine the minimum inhibitory concentration (MIC) of Cu<sup>+2</sup> on mannitol-glutamate-yeast (MGY) extract medium amended with CuSO<sub>4</sub>·5H<sub>2</sub>O (CAS:7758-99-8, Merck, Darmstadt, Germany) and cycloheximide 100 µg mL<sup>-1</sup>. CuSO<sub>4</sub>·5H<sub>2</sub>O and cycloheximide were added to the autoclaved medium immediately before plating at 50 °C. The tested Cu<sup>+2</sup> concentrations were 0.0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2 and 3.6 mM. Some 100 µL were taken from liquid cultures of the *Pseudomonas* isolates previously incubated for 48 h at 26 °C in King's medium B, and evenly plated on the MGY medium. A bacterial isolate was considered resistant when its MIC was equal to or higher than 0.8 mM of copper sulphate (Cazorla et al., 2002). Three replicates were evaluated for each treatment of [bacterial isolated] × [Cu<sup>+2</sup>]. The presence of the *copABCD* operon and their two-component regulatory genes *copR* and *copS* were evaluated according to (Petriccione et al., 2017). Table 1 shows the PCR primers for *copR*, *copS*, *copA*, *copB*, *copC* and *copD* genes.

## RESULTS

### Bacteria isolation and characterization

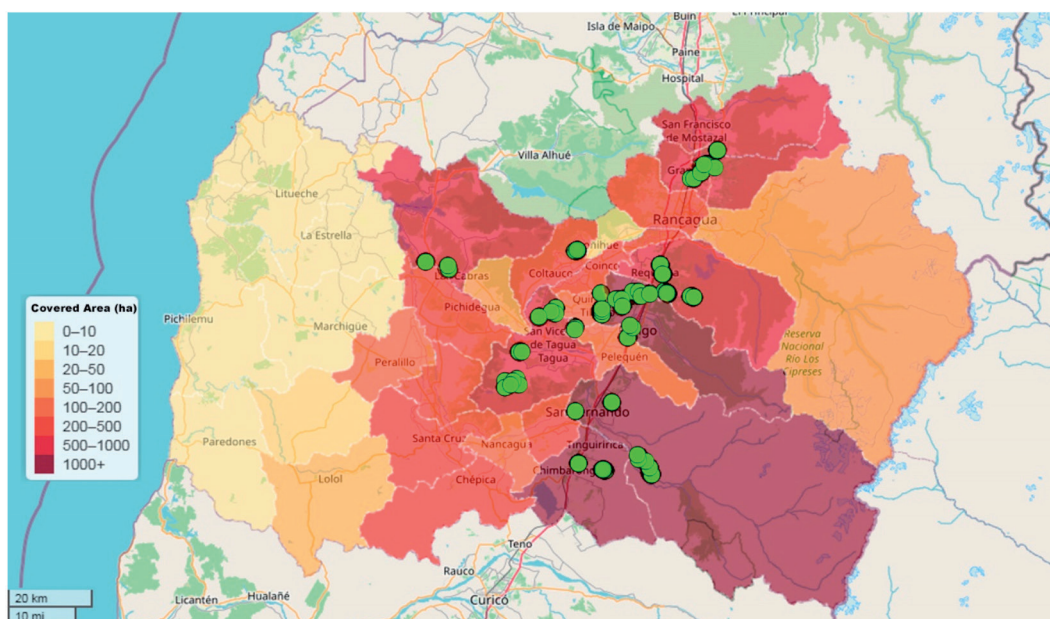
The sampling points were distributed in the main area of cherry production in the O'Higgins Region of Chile (Figure 1). A total of 79 bacteria isolates were successfully isolated from 18 commercial sweet cherry orchards (Table 2). The number of bacterial isolates obtained from specific orchards ranged from 1 to 28. Most isolates came from the sites of Rengo, San Fernando and San Vicente, with 10, 22 and 28 bacterial isolates, respectively. The cherry samples represented eight varieties, but 'Royal Dawn', 'Lapins', 'Santina' and 'Bing' were the most represented, with 11, 11, 13 and 26 bacterial isolates, respectively. One sample was from Los Lagos Region, which is 800 km south of the O'Higgins Region.

Only 21 bacterial isolates showed fluorescence under UV light (Table 2). All the bacterial isolates amplified the 16S *rRNA* gene fragment indicating the good quality of the DNA obtained. Regarding the PCR product of *rpoD* and *cts* genes, 33 isolates showed amplicons with their respective expected size for *Pseudomonas* genus, but 8 and 5 isolates generated amplicons only for the *rpoD* and *cts* genes, respectively. All of 21 fluorescent isolates showed the *cts* gene amplicon, and two did not amplify the product of *rpoD* gene. Twenty-five isolates that amplified either or both amplicons of the *rpoD* and *cts* genes were not fluorescent. Thirty-four bacterial isolates did not amplify the right fragment and/or did not show any amplicons (Table 2).

Putative Pss strains were identified by the presence of the *syrB* and *syrD* genes, which are associated with the ability to produce toxins such as syringomycin (Sorensen et al., 1998). Six isolates amplified both the 752-pb from *syrB* and 446-pb from *syrD* fragments. Four isolates produced the amplicon from the *syrD* gene, but not the *syrB* amplicon (Table 2).



**Figure 1. Area of sampling of bacteria associated to bacterial canker in commercial sweet cherry orchards from 11 sites within the O'Higgins Region, during 2017 and 2018 seasons.**



**Table 2. Bacterial isolates associated with canker disease from sweet cherry orchards in O'Higgins Region.**

Isolate ID	Place of isolation	Sweet cherry cultivar	Fluorescence	Gene presence based on PCR					MIC (mM)	<i>cop</i> gene cluster
				<i>rRNA 16s</i>	<i>rpoD</i>	<i>cts</i>	<i>syrb</i>	<i>syrd</i>		
A1M3	Rengo	ni	+	+	+	+	+	+	1.6	A
A1M25	Los Lagos Region	ni	+	+	+	+	+	+	0.8	A
A1M70	San Fernando	Royal Dawn	-	+	-	-	-	-	0.0	0
A1M71	San Fernando	Royal Dawn	-	+	-	-	-	-	0.0	0
A2M71	San Fernando	Royal Dawn	-	+	-	-	-	-	3.2	0
A1M74	San Fernando	Bing	-	+	-	-	-	-	3.2	0
A1M83	Graneros	Bing	-	+	-	-	-	-	1.2	0
A1M90	San Fernando	Lapins	-	+	-	-	-	-	3.6	0
A1M93	San Fernando	Lapins	-	+	+	-	-	+	2.0	0
A1M94	San Fernando	Bing	-	+	+	+	-	-	3.6	A
A1M95	San Fernando	Bing	+	+	+	+	-	-	2.0	0
A1M96	San Fernando	Bing	+	+	+	+	-	-	3.2	0
A2M96	San Fernando	Bing	+	+	+	+	-	-	3.6	0
A1M97	San Fernando	Bing	-	+	-	-	-	-	2.4	0
A1M98	San Vicente	Santina	-	+	-	-	-	-	2.0	0
A1M100	San Vicente	Santina	-	+	-	-	-	-	2.4	0
A1M103	San Vicente	Sweet heart	-	+	-	-	-	-	3.6	0
A2M103	San Vicente	Sweet heart	+	+	+	+	-	-	2.4	0
A1M104	San Vicente	Sweet heart	-	+	-	-	-	-	1.2	0
A1M109	San Vicente	Rainier	-	+	+	-	-	+	3.2	0
A1M110	San Vicente	Santina	-	+	-	-	-	-	1.2	0
A3M110	San Vicente	Santina	-	+	+	-	-	+	3.2	ARS
A1M111	San Vicente	Santina	-	+	-	-	-	-	3.6	0
A1M112	San Vicente	Santina	-	+	-	-	-	-	0.8	0
A1M113	San Vicente	Santina	-	+	+	-	-	-	3.2	0
A2M113	San Vicente	Santina	-	+	-	-	-	-	2.4	0
A1M115	San Vicente	Royal Dawn	-	+	-	-	-	-	0.8	0
A1M118	San Vicente	Lapins	-	+	+	+	-	-	3.6	A
A1M121	Olivar	ni	-	+	-	-	-	-	0.8	0
A2M121	Olivar	ni	-	+	+	-	-	-	3.2	0
A1M125	Codegua	Regina	-	+	-	-	-	-	2.4	0
A1M126	Rengo	Lapins	-	+	+	+	-	-	3.6	0
A2M126	Rengo	Lapins	+	+	-	+	-	-	3.6	0

Continuation Table 2.

Isolate ID	Place of isolation	Sweet cherry cultivar	Fluorescence	Gene presence based on PCR					MIC (mM)	<i>cop</i> gene cluster
				<i>rRNA 16s</i>	<i>rpoD</i>	<i>cts</i>	<i>syrB</i>	<i>syrD</i>		
A1M127	Rengo	Bing	-	+	+	+	-	-	2.8	0
A1M129	Rengo	Lapins	+	+	+	+	+	+	2.8	RS
A2M129	Rengo	Lapins	+	+	+	+	-	-	3.6	0
A1M131	Rengo	Royal Dawn	-	+	+	+	-	-	3.6	0
A1M132	Rengo	Sweet Heart	-	+	+	+	-	-	3.6	0
A1M135	Requinoa	Santina	+	+	+	+	-	-	2.8	RS
A1M137	Quinta de Tilcoco	Bing	-	+	+	-	-	-	2.4	0
A1M139	San Fernando	Bing	-	+	-	-	-	-	1.2	0
A1M140	San Fernando	Bing	+	+	+	+	+	+	2.4	ARS
A1M146	San Fernando	Bing	-	+	-	-	-	-	2.8	0
A2M146	San Fernando	Bing	-	+	+	-	-	-	0.8	D
A1M151	Coltauco	Sweet Heart	-	+	-	-	-	-	0.8	0
A1M152	Coltauco	Santina	-	+	+	+	-	-	3.2	0
A1M156	Coltauco	Santina	-	+	-	-	-	-	2.0	0
A1M157	San Vicente	Royal Dawn	+	+	+	+	-	-	3.6	0
A1M158	San Vicente	Royal Dawn	+	+	+	+	-	+	3.6	0
A1M159	San Vicente	Royal Dawn	-	+	-	-	-	-	2.0	0
A2M159	San Vicente	Royal Dawn	-	+	+	+	-	-	3.6	0
A1M160	San Vicente	Royal Dawn	+	+	+	+	-	-	1.2	0
A1M163	San Vicente	Bing	+	+	+	+	+	+	0.8	A
A2M163	San Vicente	Bing	-	+	+	+	-	-	3.6	0
A1M164	San Vicente	Bing	+	+	+	+	-	-	1.6	A
A2M164	San Vicente	Bing	-	+	-	-	-	-	0.8	0
A1M165	San Vicente	Bing	-	+	-	-	-	-	0.8	0
A1M166	San Vicente	Bing	-	+	+	-	-	-	0.8	0
A2M166	San Vicente	Bing	-	+	-	+	-	-	3.6	A
A1M167	San Vicente	Bing	-	+	-	-	-	-	0.8	0
A1M168	Rengo	Bing	+	+	-	+	-	-	3.6	0
A1M169	Rengo	Bing	-	+	-	-	-	-	3.2	0
A1M170	Malloa	Bing	-	+	-	-	-	-	3.2	0
A1M171	Malloa	Stella	+	+	+	+	+	+	2.4	A
A1M173	Malloa	Santina	-	+	-	-	-	-	0.4	0
A1M175	Malloa	Santina	-	+	-	+	-	-	0.8	0
A1M176	Malloa	Bing	-	+	-	-	-	-	0.4	0
A2M176	Malloa	Bing	+	+	+	+	-	-	2.4	A
A1M177	Las Cabras	Lapins	-	+	-	+	-	-	3.6	0
A1M178	Las Cabras	Lapins	-	+	+	+	-	-	3.2	A
A2M178	Las Cabras	Lapins	+	+	+	+	-	-	3.6	0
A1M182	San Vicente	Royal Dawn	-	+	+	+	-	-	3.2	A
A1M183	Las Cabras	Lapins	-	+	-	-	-	-	2.8	0
A1M188	San Fernando	Lapins/Bing	-	+	+	+	-	-	3.2	0
A1M189	San Fernando	Lapins/Bing	-	+	-	-	-	-	2.0	0
A1M190	San Fernando	Lapins/Bing	-	+	+	+	-	-	3.6	0
A2M190	San Fernando	Lapins/Bing	-	+	-	-	-	-	2.8	0
A1M191	San Fernando	Lapins/Bing	+	+	+	+	-	-	3.2	0
A1M193	San Fernando	Lapins-Bing	-	+	-	-	-	-	0.0	0
A2M193	San Fernando	Lapins-Bing	-	+	+	+	-	-	3.6	A

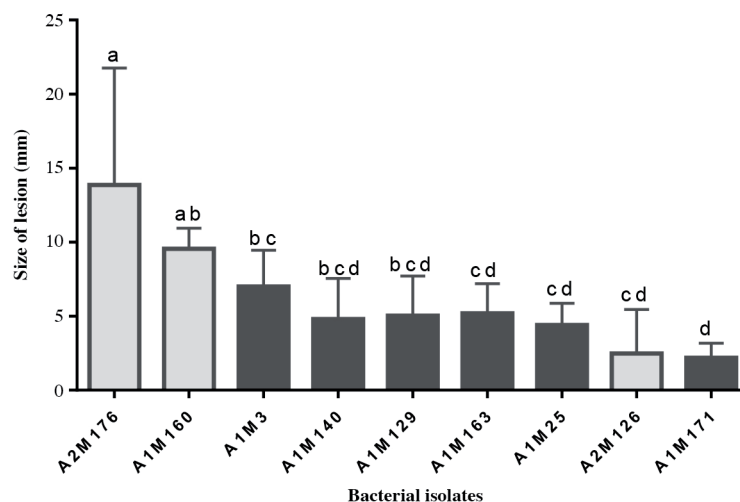
MIC: Minimum inhibitory concentration; ni: no information; +: positive-presence of features; -: negative results-lack of features.

The pathogenicity test with immature sweet cherries showed that only nine isolates produced symptoms. Six of them reproduced the typical wound caused by Pss strains, with a sunken brownish necrosis lesion (Figure 2A). Three isolates produced soft rot lesions (Figure 2B), which appeared at 3 DPI and covered most of the fruit by 10 DPI. The lesions were measured at 10 DPI and significant differences in lesion size were found among the pathogenic isolates (Figure 3). Six isolates, A1M3, A1M140, A1M129, A1M163, A1M25 and A1M171, that induced symptoms in immature cherries described for Pss strains, were positive for fluorescence under UV light and the presence of the genes *syrB* and *syrD* related to the synthesis of syringomycin (Table 2). Additional characterization with LOPAT test and ice nucleation activity confirmed the identity of these six isolates as Pss.

**Figure 2.** Symptoms observed in the pathogenicity test with immature sweet cherries at 3 d post-inoculation. Brown/black lesions caused by typical *Pseudomonas syringae* pv. *syringae* strains (a), soft rot lesion (b), and control of inoculation with only culture medium (c).



**Figure 3.** Size of lesions on immature cherries caused by pathogenic isolates by 10 d post-inoculation. Black bars represent bacterial isolates that caused brownish black lesions, like the *Pseudomonas syringae* pv. *syringae* strains. Gray bars represent the isolates that produce soft rot lesions.



An ANOVA was performed with 10 replicate data points for each isolate. Lowercase letters above the bar indicate significant groups defined by the Tukey-HSD test ( $P \leq 0.05$ ).

### Copper-resistance and cop gene cluster detection

The resistance or susceptibility to Cu was evaluated on MGY medium supplemented with  $\text{CuSO}_4 \cdot 5\text{H}_2$ . Of the 80 bacterial isolates evaluated for Cu resistance, only five had a minimum inhibitory concentration (MIC)  $< 0.8 \text{ mM Cu}^{+2}$ , which were classified as Cu-susceptible, while the other 75 exhibited different levels of Cu resistance with MIC  $> 0.8 \text{ mM Cu}^{+2}$  (Table 3). According to the criteria proposed by Cazorla et al. (2002), in this study 19 bacterial isolates fell within the range of low resistance ( $1.0$  to  $1.6 \text{ mM Cu}^{+2}$ ), 21 intermediate resistance ( $2.0$  to  $2.8 \text{ mM Cu}^{+2}$ ) and 35 high Cu resistance ( $3.2$  to  $3.6 \text{ mM Cu}^{+2}$ ).

Regarding the detection of genes present in the *copABCD* operon, 17 isolates amplified at least one of the genes (Table 2). Four bacterial isolates that showed more than one *cop* gene fell within the group of intermediate-high resistance, with MICs of  $2.4$  to  $3.2 \text{ mM Cu}^{+2}$ . The bacterial isolates identified as Pss A1M3, A1M140, A1M129, A1M163, A1M25 and A1M171 showed different degree of Cu resistance with MICs of  $0.8$  to  $2.8 \text{ mM Cu}^{+2}$ . At least one gene was detected from the *copABCD* operon associated with Cu resistance in Pss bacteria (Table 4).

**Table 3. Minimum inhibitory concentration (MIC) of bacterial isolates from cherry trees to copper sulfate on MGY medium.**

Site of isolation	MIC (mM) interval for copper sulfate				Total
	Sensitive		Resistant		
	< 0.8 mM	0.8-1.6 mM	2.0-2.8 mM	3.2-3.6 mM	
Codegua	-	1	-	-	1
Coltauco	-	-	2	1	3
Graneros	-	1	-	-	1
Las Cabras	-	1	-	3	4
Malloa	-	-	3	3	6
Olivar	-	2	-	-	2
Quinta de Tilcoco	-	1	-	-	1
Rengo	-	3	3	4	10
Requinoa	1	-	-	-	1
San Fernando	1	4	4	13	22
San Vicente	3	6	9	10	28
Los Lagos Region	-	-	-	1	1
Total	5	19	21	35	80

**Table 4. *Pseudomonas syringae* pv. *syringae* isolated in this study.**

Isolate ID	Site of isolation	Fluorescence	LOPAT	Ice nucleation activity	Gene presence based on PCR amplification					Pathogenicity	MIC (mM)	<i>cop</i> gene cluster detection
					<i>rRNA 16s</i>	<i>rpoD</i>	<i>cts</i>	<i>syrB</i>	<i>syrD</i>			
A1M129	San Vicente	+	+	+	+	+	+	+	+	+	2.8	RS
A1M140	San Vicente	+	+	+	+	+	+	+	+	+	2.4	ARS
A1M163	Olivar	+	+	+	+	+	+	+	+	+	0.8	A
A1M171	Rengo	+	+	+	+	+	+	+	+	+	2.4	A
A1M25	Los Lagos Region	+	+	+	+	+	+	+	+	+	0.8	A
A1M3	San Vicente	+	+	+	+	+	+	+	+	+	1.6	A

MIC: Minimum inhibitory concentration.

## DISCUSSION

The 79 bacterial isolates obtained from sweet cherry tree tissue symptomatic of bacterial canker is a representative sample of the bacterial community associated with canker disease in orchards in the O'Higgins Region. Obtained during the 2017 and 2018 growing seasons, over 93% of the bacterial isolates evidenced some degree of resistance to Cu<sup>+2</sup>. According to the criteria of Cazorla et al. (2002), 24%, 26% and 44% of isolates respectively presented low, intermediate and high levels of resistance to copper. In general, bacteria in agricultural areas with natural and/or industrial sources of Cu present some degree of resistance to Cu. This has been reported in Cu producing countries like China, Indonesia, Russia, Zambia, and Australia (Hao et al., 2011). There have been Cu mining operations in the O'Higgins Region for more than 104 yr, and intensive agriculture for approximately 50 yr. Hence, soils in the region are highly contaminated with Cu (De Gregori et al., 2003; Burgos-Ortiz, 2017). Bacterial isolates from Cu-contaminated soil with Cu resistance have been reported in central Chile belonging to the genera *Sphingomonas*, *Stenotrophomonas* and *Arthrobacter* (Altimira et al., 2012). The present research is the first report that evidence high levels of Cu resistance among bacterial communities associated with canker disease in sweet cherry trees from orchards in the O'Higgins Region, including one Pss isolate from Los Lagos Region. High levels of Cu contamination in agricultural soils, from natural sources, mining and/or frequent use of CBAC to control plant diseases, is a rising concern, not only because of bacterial Cu resistance, but also because the accumulation of Cu affects other soil microorganisms and can cause stress on plants, affecting productivity and quality (Nagajyoti et al., 2010). Sharafi et al. (2017) reported the strong effect of Cu on pollen germination and tube growth in 10 sweet cherry cultivars. As well, Chilean cherry exports can be seriously compromised in the future by regulations in importing countries regarding trace elements of Cu in agricultural products (Lamichhane et al., 2018).

A rapid and accurate method is needed to identify *P. syringae* isolates. LOPAT tests have proven useful to identify *P. syringae* pathovars from other fluorescent *Pseudomonas* (Hildebrand et al., 1982). Nevertheless, these methods are time-consuming and do not always give clear results. On the other hand, *syrB* and *syrD* genes are conserved among a



spectrum of Pss strains and do not occur in the genome of other pathovars of *P. syringae* (Quigley and Gross, 1994). The *syrB* gene product appears to function in toxin synthesis and the SyrD protein is embedded in the cytoplasmic membrane and functions in secretion of syringomycin (Quigley and Gross, 1994). Several studies have reported that detection of the *syrB* gene is a useful and rapid method to identify Pss strains (Sorensen et al., 1998; Kaluzna et al., 2010).

The laboratory-based tests of inoculation in immature fruits distinguished pathogens from nonpathogenic *P. syringae* on sweet cherry. Nine isolates produced symptoms in immature sweet cherries, but only six of them produced the typical wound generated by Pss strains, with sunken black-brown necrosis lesions that were later identified as Pss by the presence of the *syrB* and *syrD* genes related to syringomycin synthesis (Sorensen et al., 1998). It is likely that the other three isolates that produced soft rot lesions are different *Pseudomonas* species such as *P. viridiflava*, which also is described as pathogenic in sweet cherry (Konavko et al., 2014). These results were consistent with LOPAT characterization and ice nucleation activity.

All six Pss isolates presented resistance to Cu<sup>2+</sup>, belonging to the groups of low (three isolates) and intermediate (three isolates) resistance. All of them amplified at least one PCR amplicon of detection of *copABCD* operon that is usually plasmid-borne (Cazorla et al., 2002; Altimira et al., 2012; Gutiérrez-Barranquero et al., 2013). This operon explains the widespread occurrence of this trait among distantly related bacterial species and genera (Sundin 1989; Cazorla et al., 2002), and our research suggests this operon also appears as the genetic determinant of Cu resistance in the Pss population associated with bacterial canker in sweet cherry in the O'Higgins Region. Another 11 bacterial isolates identified as *Pseudomonas*, but not Pss, also evidenced the presence of *copABCD* operon, belonging to the groups of low (one isolate), intermediate (three isolates) and high Cu resistance (seven isolates). However, most bacterial isolates identified as Cu-resistant did not amplify any PCR marker of *copABCD* operon, including two pathogenic bacterial (likely *P. viridiflava*), suggesting that there are genetic determinants other than *copABCD* operon, or they are sufficiently different to not be detected with the specific PCR markers (Petriccione et al., 2017) used in this research. Indeed, it is known that bacteria can protect themselves from the negative effects of Cu by several homeostasis or tolerance mechanisms such as transmembrane Cu exporting, Cu sequestration by metallothioneins and oxidation of Cu<sup>+</sup> by multicopper oxidases (Ladomersky and Petris, 2015). In the case of the *copABCD* operon identified in Pss, it is known that coded proteins occur in the periplasmic and outer membrane of the cell wall that confers Cu resistance by sequestering the metal outside the cytoplasm (Cha and Cooksey, 1991). Recently, Husseini and Akköprü (2020) studied the mechanisms of Cu resistance in different *P. syringae* pathovars isolated from stone fruit trees, and found the presence of *copABCD* genes and the ability to produce siderophores, but not detected other mechanisms such as *cusA* gene. The role that plays the bacterial community associate with the bacterial canker disease in sweet cherry need to be addressed in future research, both in term of its role in the disease development and Cu resistance.

The Pss isolates produced different sized lesions in immature sweet cherries, and had different degrees of Cu resistance and distinct *copABCD* operon detected-profiles (ARS, RS and A genotypes). This strongly suggests that there is genetic diversity in the Pss population that produces the canker disease bacteria. Interestingly, the Pss isolate A1M25 from the Los Lagos Region, which is 800 km to the south of O'Higgins Region, showed Cu resistance (MIC 0.8 mM) and the marker for the Cu-resistance protein CopA of the *copABCD* operon. There are no mining operations in the Los Lagos Region, nor is there a history of intensive application of Cu-based antimicrobial compounds (CBAC). Since sweet cherry production is just beginning in the Los Lagos Region, it is likely that Cu resistance was brought into the region with plants from nurseries in the O'Higgins Region.

The high Cu resistance of bacterial associated with canker disease in sweet cherry orchards in the O'Higgins Region indicates that the amount of CBACs applied needs to be reduced and that integrated pest management (IPM) strategies need to be applied (France and Millas, 2020). These include among others, pruning, calibrating and optimizing sprayers, and timing product application in accordance with the growth dynamics of epiphytic Pss populations, weather conditions, and varietal susceptibility (France and Millas, 2020). However, in agricultural areas with heavy Cu contamination and populations of Cu-resistant Pss, alternative products should be used to control the bacterial canker in sweet cherry orchards (Osorio et al., 2020).

Few alternative products based on biological control agents (BCAs) have been registered in Chile to control bacterial canker in sweet cherry ([www.sag.gob.cl](http://www.sag.gob.cl), March 2020). Most of these contain antagonist microorganisms such as strains of *Bacillus subtilis* (Serenade ASO, Bayer AG, Leverkusen, Germany) and mixtures of native strain of *Bionectria ochroleuca/Hypocrea virens/Bacillus licheniformis* (Coraza, Bio Insumos Nativa, Maule, Chile). These products performed well in

controlling trunk and branch cankers in orchards in the O'Higgins Region (Osorio et al., 2020), but the effect on Pss epiphytic populations was not clear. Most reports of the Chilean Society of Phytopathology (<http://www.sochifit.cl/>) from 2010 to 2018 evaluated new products, some of which are based on BCAs to control bacterial canker disease in sweet cherry. The BCAs are being actively researched worldwide to control several bacterial diseases (Sundin et al., 2016). Hence, alternatives to CBACs to control bacterial canker in sweet cherry can be expected in the near future. New products based on microorganisms with antagonism to Pss, small antimicrobial peptides (AMPs; Breen et al., 2015), bacteriophage (Rabiey et al., 2020), inducer of systemic acquired resistance (SAR; Gao et al., 2015) are being evaluated to control bacterial canker in *Prunus* trees.

Other approaches to control bacterial diseases have emerged based on knowledge generated by research in molecular biology and the pathogenesis of plant-pathogenic bacteria, which aim to develop products that alter the functionality of the type three secretion system (T3SS), inhibit the formation of bacterial biofilms and/or quorum sensing (QS) (Lu et al., 2019). The T3SS is a needle-like apparatus that enables pathogens to translocate effector proteins directly from the bacterial cytoplasm to host cell cytoplasm. Biofilm formation provides bacteria with effective protection from environmental stresses, antimicrobial drugs and host defense mechanisms, and is a critical virulence factor of many pathogenic plant bacteria. Quorum sensing (QS) is cell-to-cell communication involved in the regulation of various traits in response to cell density via extracellular signaling molecules. Nanoparticles (NPs) have also been described as an alternative to control bacterial disease. NPs have unique physical and chemical properties, are highly reactive and have unique interactions with biological systems that make them excellent antimicrobials, as well as having properties that make them ideal carrier/delivery systems for other antimicrobials (Sundin et al., 2016).

A better understanding of the environmental conditions that determine the development of the disease will also help in implementing more effective IPM strategies to control the bacterial canker in sweet cherry. In this regard, there have been only two reports, Latorre et al. (2002), who studied the effects of temperature, free moisture duration and inoculum concentration on infection of sweet cherry by Pss in immature fruits and 1-yr-old twigs, and Donoso et al. (2018), who studied the dynamics of epiphytic populations of Pss in the Maule Region. Recently, Osorio et al. (2020) evaluated the severity of the disease in 23 sweet cherry varieties in the O'Higgins Region during the 2016, 2017 and 2018 growing seasons. The multifactorial analysis of the data clearly established that the severity of the bacterial canker mainly depends on the variety and the occurrence of frost at the time of flowering (Osorio et al., 2020). Other factors, such as rootstock, plant age, and the conduction system, also affect the severity of the disease (Osorio et al., 2020).

Host plant resistance is the most desirable element to develop IPM strategies (Dara, 2019). Resistant plants reduce the need for pesticides like CBAC, and other products that are expensive and pose risks for humans and animals (Dara, 2019). It also reduces production costs by reducing the need for labor, which is increasingly expensive (Dara, 2019). However, breeding new varieties of sweet cherry is a long-term process. After successful crossing, germination and planting, the selection and evaluation can take as much as 20 yr (Quero-García et al., 2019).

## CONCLUSIONS

Around 95% of bacteria community associated with bacterial canker symptoms in sweet cherry from the production area of O'Higgins Region of Chile showed Cu resistance determined as the minimum inhibitory concentration using CuSO<sub>4</sub>.

Those bacterial isolates identified as *Pseudomonas syringae* pv. *syringae* (Pss), presented different degrees of Cu resistance. They presented at least one feature of the *copABCD* operon with three genotypes (ARS, RS or A). As well, they produced significant lesion sizes in immature sweet cherry fruits. This strongly suggests that there is genetic diversity in Pss population that produces the sweet cherry bacterial canker disease in the O'Higgins Region.

This information on the status of the pathogen Pss within its bacterial community in terms of pathogenicity and Cu resistance will facilitate the development of alternative integrated pest management strategies to control this important disease.

## ACKNOWLEDGEMENTS

This work was funded by Chilean Ministry of Agriculture Grant "Proyecto Núcleo P15-16" and FIC-R O'Higgins IDI 30474707-0. Proyecto Anillo ACTO190001.

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