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Antimicrobial Multiresistant Phenotypes of Genetically Diverse *Pseudomonas* spp. Isolates Associated with Tomato Plants in Chilean Orchards

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Abstract: Tomatoes are susceptible to bacterial diseases, mainly related to some *Pseudomonas syringae* pathovars. Many *Pseudomonas* species are considered innocuous, but some have shown the ability to opportunistically infect tomato plants. Antimicrobial compounds have been used to control pathogenic organisms, and this can lead to environmental selection of phenotypically resistant bacteria. We assessed the diversity of *Pseudomonas* species associated with tomato plants from Chilean orchards and analyzed antimicrobial resistance among the isolated strains. A total of 64 *Pseudomonas* isolates (*P. syringae*, *P. viridiflava*, *P. fluorescens*, *P. koreensis*, *P. gessardii*, and *P. azotoformans*) were evaluated for their phenotypic resistance to seven antimicrobial compounds, including copper, streptomycin, and five other antibiotics typically not used in agriculture. The results showed that 95%, 86%, 70%, 53%, 45%, and 1.6% of the isolates were resistant to rifampin, ampicillin, copper, chloramphenicol, streptomycin, and tetracycline, respectively, with no isolates being resistant to gentamicin. A total of 96.9% of *Pseudomonas* isolates exhibited a multiresistant phenotype to at least two of the antimicrobials tested. The most frequent multiresistance phenotype was Cu-Str-Amp-Cm-Rif (23.4%). The presence of *Pseudomonas* strains tolerant to conventional bactericides, metals, and other antimicrobials makes these bacteria an emerging threat to the agriculture industry and to human health.

Keywords: *Pseudomonas* spp.; phytopathogen; antimicrobial resistance; antibiotics; multiresistance

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1. Introduction

The tomato (*Solanum lycopersicum* L.) is the most cultivated vegetable worldwide, covering five million hectares, with production of over 180 million tons in 2018. In Chile, the tomato crop has the third largest planted area, exceeding 15,000 hectares, with 951,000 tons produced per year [1]. The Chilean tomato industry has grown approximately 25% in the last decade. However, a range of factors limit this growth, such as diseases of viral, fungal, or bacterial origin, with the latter being responsible for considerable annual production losses [2].

Tomato plants harbor diverse bacterial communities in their rhizosphere, phyllosphere, and endosphere that play crucial roles in plant health and growth. In a previous study of the microbiome associated with tomato plants, Ottesen et al. (2013) [3] showed that the most frequently observed bacterial taxa across the plant phyllosphere (leaves, flowers, and stems) are those belonging to the genera *Pseudomonas* and *Xanthomonas* [3]. Another

study of tomato plant bacterial communities revealed the prevalence of *Pseudomonas* and *Acinetobacter* in the rhizosphere; and *Acinetobacter*, *Enterobacter*, and *Pseudomonas* in the endosphere, phyllosphere, and rhizosphere (roots, stems, and leaves) [4]. Additionally, a study on the leaf-associated microbiomes of grafted tomato plants showed that the genera *Sphingomonas*, *Methylobacterium*, and *Pseudomonas* were often seen across the rootstock (rhizosphere) of the varieties examined [5]. Thus, multiple studies have shown that the genus *Pseudomonas* is common in the microbiomes of tomato plants.

Currently, there are 247 recognized species of the genus *Pseudomonas* [6]. Many of these are distributed worldwide both in the soil and on the aerial surfaces of plants (epiphytes). *Pseudomonas* spp. are aerobic, non-spore-forming, Gram-negative, rod-shaped bacteria with one or more polar flagella. Some *Pseudomonas* species are pathogenic, with the non-pathogenic ones acting as commensals with no known effect on their host plant. However, other species, such as *P. fluorescens*, *P. mohnii,* and *P. plecoglossicida,* establish a mutualistic relationship in which the host provides nutrients and refuge, and the bacteria promote plant growth and enhance resistance against insects, metals, and pathogens [4]. Among the pathogenic *Pseudomonas* associated with tomato plants, *P. syringae* has been studied most due to its high destructive potential. However, other species of the genus *Pseudomonas* present in the soil or phyllosphere are considered pathogenic, such as *Pseudomonas corrugata* and *Pseudomonas viridiflava* [7,8], while still others, generally considered innocuous, can be opportunistically involved in tomato diseases, such as some strains of *Pseudomonas fluorescens* [9,10].

Since the development of intensive agricultural production, the agri-food industry has benefitted from the availability of antimicrobial compounds for food and animal production and crop protection. Agricultural antimicrobials such as copper and streptomycin have been used for decades to control infections by phytopathogens [11,12]. However, the improper and excessive use of chemical control has fostered the selection of bacterial strains that are resistant to bactericidal compounds, diminishing the success of efforts to control phytopathogens. This has led to increased doses or frequency of agrochemical applications, thus contributing the increase in levels of resistance in both pathogenic and other environmental bacteria (understood as bacteria present in the soil, water, air, and sediments covering the planet, including animals and plants that inhabit these areas) [11]. Since the mid-1980s, there has been an increase in the number of reports on copper tolerance in a wide range of bacteria important to the agricultural environment, including pathogenic and environmental strains such as *P. syringae*, *P. fluorescens,* and *P. putida* [11,13–15]. Additionally, the prolonged use of the antibiotic streptomycin in agriculture has given rise to streptomycin resistance in plant pathogens. Bacterial strains of *P. syringae* pv. *lachrymans*, *P. syringae* pv. *papulans*, *P. syringae* pv. *syringae*, and *P. syringae* pv. *Actinidae*, as well *Erwinia amylovora, Erwinia carotovora,* and *Xanthomonas campestris*, were resistant to streptomycin and have been isolated in North and South America [12,16,17].

Currently, bacterial resistance to copper and streptomycin in agricultural environments is an increasingly common phenomenon that has been described in fruit trees and annual crops [11]. This situation is exacerbated by evidence suggesting the transfer of resistance genes between bacteria [17,18]. Resistance genes are transferred to new hosts by horizontal gene transfer, conferring multiresistance to phytopathogenic bacteria [19]. Moreover, several studies support the idea that environmental bacteria, including those associated to edible plants, play a role as vector of antimicrobial resistance genes (ARGs), mainly via their mobilization trough the food chain [20]. An example of this is the presence of antimicrobial resistant bacteria in fresh fruits and vegetables (including tomato) for human consumption [21–23]. Members of genera such as *Enterobacter, Acinetobacter, Pseudomonas, Staphylococcus, Burkholderia, Serratia, Stenotrophomonas,* and *Bacillus* often associated with crops, may harbor antibiotic-resistance genes [24]. Moreover, the study of Sun et al. (2021) in fresh tomatoes ready for human consumption in the Chinese market, detected a total of 191 ARGs and 10 mobile genetic elements (MGEs) on fresh tomato surfaces [23]. Their results indicated that fifteen bacterial families might be the potential hosts of ARGs. These

results are a call to pay more attention to ecological environment impacts of ARGs and ARB on the surfaces of vegetable or fruit.

Many nurseries alternate the spraying of copper and streptomycin or combine them to reduce the emergence of resistant strains and avoid copper phytotoxicity, but this has been insufficiently effective due to the selection of multiresistant bacterial strains [25,26]. Currently, concern is growing with regard to the selection of bacteria resistant to both antibiotics and metals (such as copper) due to their excessive agricultural use [25,26]. It has been shown that the presence of high concentrations of metals favors the co-selection of resistance to antibiotics [26]. An example of this is the case described in nurseries of the Pacific Northwest of United States, where research detected that 24% of *P. syringae* pvs. isolates were resistant to both copper and streptomycin and presented the characteristic genes of resistance to these compounds [27].

Although, the excessive use of agrochemicals has been pointed out by different authors as the main cause of resistance development, other possible sources of antibiotic/metal resistance need to be considered. Several studies demonstrate that plant disease control agents such as antibiotics or copper, also affect the native phyllosphere and soil microflora and further indicated that antibiotic resistance genes can be selected in epiphytic bacteria in antibiotic-sprayed plant habitats and could provide a route of acquisition by plant pathogens [12]. Moreover, it must be considered the effect of environment contamination with antimicrobials, for example, as a consequence of the use of manure in soil or reuse of wastewater for irrigation. Different studies argued that bacteria exposed to human or animal-derived biological contamination may susceptible to acquire resistance genetic determinants under selective pressure [24] and must be considered in the evaluation of risk factors contributing to the global spread of antimicrobial resistance [20].

Hwang et al. (2005) focused on *Pseudomonas syringae* isolated from different crops including tomato, between years 1935–1998 and from different countries including USA, UK, Japan, Canada, Switzerland, Zimbabwe, Ethiopia, Yugoslavia, Greece. Overall, the results show, that from a total of 95 strains, 75%, 58%, 38%, 16%, 8%, and 1% showed resistant phenotypes against copper, ampicillin, chloramphenicol, rifampicin, streptomycin, and kanamycin–tetracycline, respectively [28]. This demonstrated that the problem of antimicrobial resistance is present worldwide even in plant-associated bacteria and since at least 20 years ago. Recent studies have confirmed that this is a problem common for different bacterial species; for example, *Escherichia coli* strains isolated from the jalapeño pepper, tomato, and cantaloupe farm environments in Northeast Mexico presented resistance to more than five antibiotics [29]. Finally, in relation to the seriousness of the antimicrobial resistance problem in agricultural bacteria compared with human pathogens, we share the opinion of Scaccia et al. (2021) that, under the One-Health concept (humans, animals, and environment), the environment contamination with antibiotic-resistant bacteria cannot be dissociated from its potential transmission to humans [24]. Thus, we consider that the presence of environmental bacteria resistant to antibiotics should be considered a risk as serious as antibiotic resistance in human pathogens.

In this context, agricultural environments are considered an important reservoir of antimicrobial resistance genes in nature and a potential source for antimicrobial resistance in bacteria, including those pathogenic for animals and humans. Hence, the growing concern regarding the accumulation of antimicrobial resistance genes in agricultural ecosystems (soil, water, plant remains, composted plant tissue, and crops, among others) and their potential spread to other environments [30].

Earlier studies have shown that antimicrobial resistance is present in agricultural environments of Chile [31–33]. However, there are a limited number of studies on this topic in bacteria associated with these agricultural crops, with this study being the first associated with tomato crops in agricultural environments of our country. Thus, the objective of this study was to evaluate the phenotypic resistance to agrochemicals and to antimicrobials used in other fields, such as human medicine in bacterial strains associated with tomato crops,

especially strains of the genus *Pseudomonas*, including both pathogenic and environmental strains that may act as reservoirs of resistance genes in Chilean orchards.

The antimicrobials analyzed in this study were as follows: copper, a metal that is cytotoxic at high concentrations due to the generation of reactive intermediaries that can cause DNA damage, degrade lipids, and disrupt normal protein function leading to cell death [34]; streptomycin, an aminoglycoside antibiotic commonly used in agriculture with bactericidal interference on protein synthesis by inactivation of the 30S ribosome; ampicillin, which belongs to the penicillin group of beta-lactam antibiotics that inactivate penicillin-binding proteins, thereby inhibiting cell wall biosynthesis; rifampin, of the rifamycin class of antibiotics, which target the subunit of RNA polymerase II, thereby inhibiting transcription initiation; chloramphenicol, a broad spectrum antibiotic that binds to the 70S ribosome and inhibits the peptidyl transferase reaction during translation; gentamycin, an aminoglycoside antibiotic which inhibits protein synthesis by targeting the 30S ribosome; and tetracycline, which inhibits chain elongation during protein synthesis by blocking aminoacyl tRNA binding at the A site [28]. These antibiotics were chosen due to their use in agriculture (streptomycin and gentamycin) or in human/veterinary medicine (ampicillin, chloramphenicol, rifampin, and tetracycline) [35–38]. We chose to work with antibiotics belonging to different classes with varied mechanisms of action. Additionally, we considered information from previous studies reporting environmental or plant/human pathogenic *Pseudomonas* resistance to antimicrobials [28,35,37].

2. Materials and Methods

2.1. Bacterial Isolation and Culture Conditions

A total of 58 strains of *Pseudomonas* spp. were isolated between April and December 2018, and six additional isolates were obtained from samples taken in November 2020 and March 2021, providing a total of 64 isolates included in this study. Unless otherwise specified, bacterial isolates were recovered from the vegetable tissue of tomato plants cultivated in greenhouses, open fields, and nurseries found throughout four regions of the Central Zone of Chile: Valparaíso, O'Higgins, Maule, and Bío Bío (Figure 1). These regions were chosen, as they are the most important to produce tomatoes in Chile, equivalent to more than 75% of the national productive surface [1]. Several studies have shown the ability of *Pseudomonads* to survive in the environment outside their host plants, even in non-agricultural habitats [39]. For these reasons, samples of irrigation water, soil and other vegetables grown in the same agricultural environment were included for their analysis. Details of the origin of each strain are described in Table 1.

Before processing for bacterial isolation, vegetal tissue was surface disinfected by immersion for 2 min in 5% sodium hypochlorite solution and then immersed for 5 min in sterile distilled water.

To obtain isolates, the plant samples were processed as described below: from each sample, a pool of 10 leaves (or pieces of fruit) was collected and macerated. For the maceration process, 3–5 circular pieces (approximately 1 cm in diameter) of leaf tissue or one fruit piece (approximately 1 cm^3) was mixed with 300 µL of buffered physiological water (BPW buffer) (0.14 M NaCl, 2.6 mM NaH_2PO_4 x $2H_2O$, and 7.5 mM Na_2HPO_4 x $12H_2O$). Bacterial isolation was conducted under sterile conditions by inoculating 50 µL of supernatant from each resulting mash onto solid King's B medium (KB) (2% protease peptone, 0.15% K_2PO4, 0.15% $MgSO_2$ x $7H_2O$, 1% glycerol, and 1.5% agar, pH 7) [40] and incubated at 28 °C for a period of 24 to 72 h. The initial selection of bacterial isolates was made based on the macroscopic characteristics of the colonies, such as color, shape, brightness, and mucosity. Then the genus of each sampled isolate was confirmed by sequencing the 16S rRNA genes and comparing them with sequences available in the GenBank database. The isolated strains were cultivated in solid KB medium or nutrient broth (NB) (meat extract 0.3%, peptone 0.5%) and incubated at 28 °C. For long-term maintenance, the isolated strains were cryopreserved and stored at −80 °C in nutrient broth with glycerol in a 1:1 ratio.

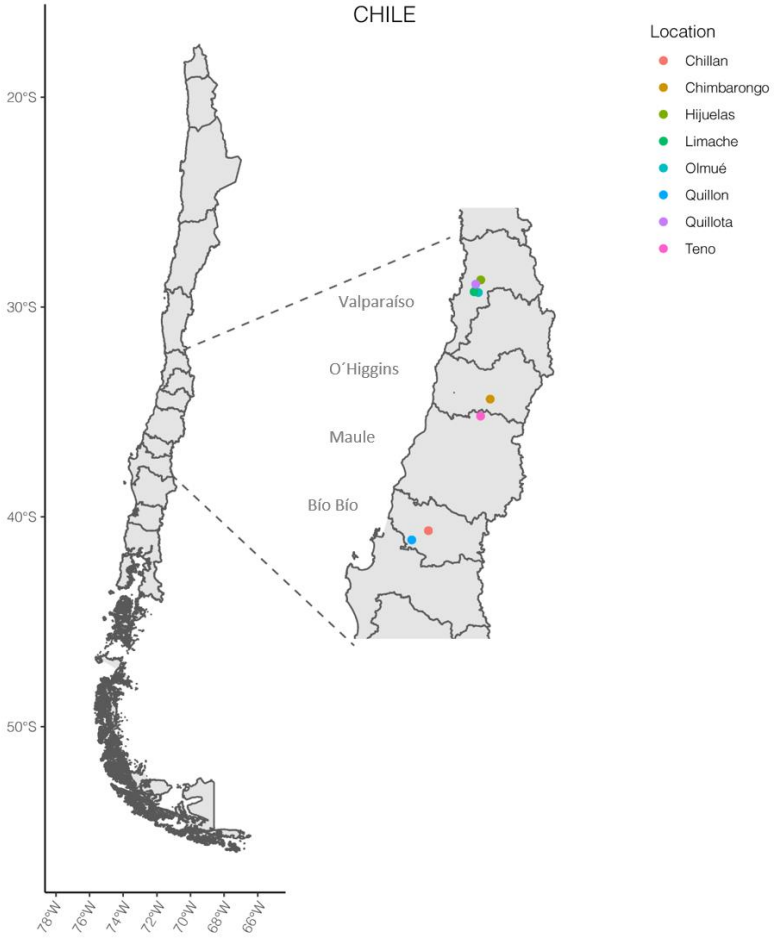

Figure 1. Geographical distribution map of sampling locations for *Pseudomonas* isolates in Chile. On the right, the central zone of the country is enlarged, and the four regions (Valparaíso, O´Higgins, Maule, and Bío Bío) show where sampled tomato-producing orchards were found. Colored points show locations of the sampled orchards inside each region.

Table 1. Bacterial species most closely related to *Pseudomonas* spp. isolates based on identity of 16S rRNA gene sequences.

Strain	Sample Origin	Plant Health Appearance [2,3]	Cultivation Modality	Región [1]/Location	Isolation Date
pA2.4	tomato plant	with signs of illness	greenhouse	V/Olmué	August 2018
pA2.5	tomato plant	with signs of illness	greenhouse	V/Olmué	August 2018
pA2.6	tomato plant	with signs of illness	greenhouse	V/Olmué	August 2018
pAI.1	tomato plant	with signs of illness	greenhouse	V/Olmué	August 2018
pAII.1	tomato plant	with signs of illness	greenhouse	V/Olmué	August 2018
pAII.2	tomato plant	with signs of illness	greenhouse	V/Olmué	August 2018
pFL13	tomato plant debris	recently dead	greenhouse	V/Limache	July 2018
pFL17	tomato plant debris	recently dead	greenhouse	V/Limache	July 2018
pFL2	tomato plant	healthy	greenhouse	V/Limache	July 2018
pFL3	tomato plant	healthy	greenhouse	V/Limache	July 2018
pFL4	tomato plant	healthy	greenhouse	V/Limache	July 2018

Table 1. *Cont.*

Strain	Sample Origin	Plant Health Appearance [2,3]	Cultivation Modality	Región [1]/Location	Isolation Date
pFL5	tomato plant	healthy	greenhouse	V/Limache	July 2018
pFL8	tomato plant	healthy	greenhouse	V/Limache	July 2018
pJV.8r	tomato plant	with signs of illness	greenhouse	V/Limache	August 2018
pJV.9r	tomato plant	with signs of illness	greenhouse	V/Limache	August 2018
pJ.1	tomato plant	with signs of illness	greenhouse	V/Limache	August 2018
pJ3.2	tomato plant	with signs of illness	greenhouse	V/Limache	August 2018
pJ4.1	tomato plant	with signs of illness	greenhouse	V/Limache	August 2018
pJ5.1	tomato plant	with signs of illness	greenhouse	V/Limache	August 2018
pJ5.2	tomato plant	with signs of illness	greenhouse	V/Limache	August 2018
pJ4.2	tomato plant	with signs of illness	greenhouse	V/Limache	August 2018
pJ6.3	tomato plant	with signs of illness	greenhouse	V/Limache	August 2018
p1.12	tomato seedling	healthy	nursery	V/Hijuelas	August 2018
p10.3	tomato seedling	healthy	nursery	V/Hijuelas	August 2018
p2.2	tomato seedling	healthy	nursery	V/Hijuelas	August 2018
p4.1	tomato seedling	healthy	nursery	V/Hijuelas	August 2018
p4.8	tomato seedling	healthy	nursery	V/Hijuelas	August 2018
p9.7	tomato seedling	healthy	nursery	V/Hijuelas	August 2018
p9.9	tomato seedling	healthy	nursery	V/Hijuelas	August 2018
p4.3	tomato seedling	with signs of illness	nursery	V/Hijuelas	August 2018
pAZ.24	tomato plant	healthy	open field	VII/Teno	December 2018
pAZ.25	tomato plant	healthy	open field	VII/Teno	December 2018
pAZ.26	tomato plant	healthy	open field	VII/Teno	December 2018
p27Ch	tomato plant	with signs of illness	open field	VIII/Chillán	April 2018
p29Ch	tomato plant	with signs of illness	open field	VIII/Chillán	April 2018
p42Ch	tomato plant	with signs of illness	open field	VIII/Chillán	April 2018
pH7.13	tomato plant	healthy	greenhouse	VIII/Quillón	December 2018
pH7.17	tomato plant	healthy	greenhouse	VIII/Quillón	December 2018
pH6.6	tomato plant	healthy	greenhouse	VIII/Quillón	December 2018
pH7.10	tomato plant	healthy	greenhouse	VIII/Quillón	December 2018
pHm27	tomato plant	healthy	greenhouse	VIII/Quillón	December 2018
pH7.14	tomato plant	healthy	greenhouse	VIII/Quillón	December 2018
pHm28	tomato plant	healthy	greenhouse	VIII/Quillón	December 2018
pH7.18	tomato plant	healthy	greenhouse	VIII/Quillón	December 2018
pH7.9	tomato plant	healthy	greenhouse	VIII/Quillón	December 2018
pH6.2	tomato plant	healthy	greenhouse	VIII/Quillón	December 2018
pH6.5	tomato plant	healthy	greenhouse	VIII/Quillón	December 2018
p22	tomato seedling	with signs of illness	nursery	V/Quillota	November 2020
p23	tomato seedling	with signs of illness	nursery	V/Hijuelas	November 2020
p24	tomato seedling	with signs of illness	nursery	V/Quillota	November 2020
p26	tomato seedling	with signs of illness	nursery	V/Hijuelas	March 2021
p27	tomato seedling	with signs of illness	nursery	V/Hijuelas	March 2021
p28	tomato seedling	with signs of illness	nursery	V/Limache	March 2021
pT11	tomato seedling	with signs of illness	nursery	VI/Chimbarongo	August 2018
pLO2.3	tobacco seedling	with signs of illness	nursery	VI/Chimbarongo	October 2018
pLO4.2	tobacco seedling	with signs of illness	nursery	VI/Chimbarongo	October 2018
pLO5.2	cucumber seedling	healthy	nursery	VI/Chimbarongo	October 2018
pl1.1	lettuce seedling	healthy	nursery	VI/Chimbarongo	August 2018
pl3.12	lettuce seedling	healthy	nursery	VI/Chimbarongo	August 2018
pl1.2	lettuce seedling	with signs of illness	nursery	VI/Chimbarongo	August 2018
pl1.3	lettuce seedling	with signs of illness	nursery	VI/Chimbarongo	August 2018
pJS4.2	soil	-	greenhouse	V/Limache	August 2018
pJS5.5	soil	-	greenhouse	V/Limache	August 2018
pAg2	irrigation water	-	greenhouse	VII/Teno	October 2018

[1] V, Valparaiso; VI, O'Higgins; VII, Maule; VIII, Bío Bío. [2] With signs of illness: dark necrotic spots (with or without a chlorotic halo) on leaves or stems, on plants that were still green. [3] Recently dead = in the final stage of the crop (prior to being discarded), corresponding to the state known as senescence.

2.2. Molecular Characterization of Bacterial Isolates

For the molecular characterization of the isolated strains, 16S rRNA gene sequencing and multilocus sequence analysis (MLSA) were conducted, as described below. The primers used in the molecular characterization protocols are listed in Table 2.

Table 2. Primers used in this study.

Primer Name	Sequence (5′-3′)	Target Gene	Tm (°C) [1]
27F	AGAGTTTGATCMTGGCTCAG	*16SrRNA*	58
1492R	TACGGYTACCTTGTTACGACTT		
cts-Fp	AGTTGATCATCGAGGGCGCWGCC	*cts*	56
cts-Rp	TGATCGGTTTGATCTCGCACGG		
acn-Fp	ACATCCCGCTGCACGCYCTGGCC	*acn*	60
acn-Rp	GTGGTGTCCTGGGAACCGACGGTG		
pgi-Fp	TGCAGGACTTCAGCATGCGCGAAGC	*pgi*	60
pgi-Rp	CGAGCCGCCCTGSGCCAGGTACCAG		
rpoD-Fp	AAGGCGARATCGAAATCGCCAAGCG	*rpoD*	63
rpoD-Rps	GGAACWKGCGCAGGAAGTCGGCACG		

[1] Temperature of annealing used for PCR amplification conditions with each primer pair.

2.3. Amplification, Sequencing and Analysis of the 16S rRNA Gene

The genomic DNA of bacterial isolates was extracted by using the Wizard® Genomic DNA Purification Kit (Promega, WI, USA) according to the manufacturer's instructions. PCR amplification of the *16S rRNA* gene was conducted by using established primers 27F and 1492R (Table 2) [41]. PCR was prepared according to the GoTaq® DNA polymerase (Promega, WI, USA) standard protocol. Amplification conditions consisted of initial denaturation for 3 min at 95 °C, followed by thirty cycles of 30 s at 95 °C, 1 min at 58 °C, and 1 min at 72 °C, plus a final extension of 7 min at 72 °C. The PCR product obtained was checked by electrophoresis in an 8% polyacrylamide gel, along with a 100 bp DNA ladder (Promega, WI, USA), following the standard running protocol (200 V, 20 min) [42]. Subsequently, the amplicons were purified and sequenced by an external service at Macrogen Inc. (Seoul, Korea). All sequences were edited and compiled by using Geneious Prime® 2021.1.1 (Biomatters Ltd., Auckland, New Zealand). Partial 16S sequences of 763 bp were obtained for each isolate. Each sequence was compared against the nonredundant nucleotide database of the NCBI (National Center for Biotechnology Information), using the BLAST tool of Geneious software. *Pseudomonas* species sequences among the first hits with at least 99% identity were considered for selection of reference sequences in later analyses.

2.4. Housekeeping Gene Sequencing and Multilocus Sequence Analysis (MLSA)

Multilocus sequence analysis was performed for the *Pseudomonas* isolates by PCR amplification and sequencing. Four housekeeping genes were sequenced: those encoding sigma Factor 70 (*rpoD*), aconitate hydratase B (*acnB*), citrate synthase (*cts*), and phosphoglucoisomerase (*pgi*) [43]. In addition, *16S rDNA* sequences were also considered in the MLSA analysis since their usefulness in this type of analysis was previously demonstrated [44–46]. To further analyze the genetic variability of the 64 isolated strains of *Pseudomonas* spp., four loci corresponding to *acnB*, *cts*, *pgi*, and *rpoD* housekeeping genes were analyzed in addition to the 16S rDNA sequences [44,47,48]. Each gene from the 64 different isolates was PCR-amplified and sequenced by using specific primers. Representative sequences from the genus *Pseudomonas* were included as references for comparative analysis, using only sequences from complete sequenced genomes (Table 3) [47,48]. Sequences from the corresponding genes of *E. coli* strain K-12 were included as an outgroup.

Table 3. Information details of strains used as reference for both 16SrRNA and MLSA analysis (complete genomes).

Species	Strain Name	Country	Access Number
Pseudomonas fluorescens	FDAARGOS 1088	Germany	NZ_CP068151.1
Pseudomonas syringae pv. *Syringae*	B728a	USA	NC_007005.1
Pseudomonas syringae pv. *tomato*	DC3000	UK	NC_004578.1
Pseudomonas syringae pv. *tomato*	T1	Canada	NZ_ABSM00000000
Pseudomonas viridiflava	CFBP 1590	France	NZ_LT855380.1
Pseudomonas putida	KT2440	Japan	NC_002947.4
Pseudomonas koreensis	BS3658	Korea	LT629687.1
Pseudomonas gessardii	BS2982	France	FNKR01000003
Pseudomonas azotoformans	LMG21611	Japan	LT629702
Escherichia coli	K-12	USA	U00096

Genomic DNA was prepared by using the Wizard® Genomic DNA Purification Kit protocol (Promega) according to the manufacturer's instructions. PCR amplification was performed on 250 ng of template DNA by using an Agilent thermal cycler. PCR was prepared according to the GoTaq® DNA polymerase (Promega) standard protocol with nucleotide concentrations of 200 µM each and primer concentrations of 1 µM. The amplification conditions consisted of initial denaturation for 3 min at 95 °C, followed by thirty cycles of amplification, with template denaturation at 94 °C for 2 min, the proper annealing temperature (Table 2) for 1 min, and extension at 72 °C for 1 min. A final extension step of 10 min at 72 °C was performed. The correct amplification and size of the amplicons were confirmed through electrophoresis in polyacrylamide gels, as described above.

The amplicons of each gene were sequenced by Macrogen, Inc. (Seoul, Korea). To establish genetic relatedness, the sequences were subjected to sequence pair distance and phylogenetic analysis, along with ten reference sequences (Table 3).

Nucleotide sequences were edited, assembled, aligned, trimmed, and compiled by using Geneious Prime® 2021.1.1 (Biomatters Ltd., Auckland, New Zealand). Since the amount of data obtained was different for each strain, all sequences were trimmed to include only those regions for which we had data for all strains. Sequences from each locus were aligned by using Geneious alignment default parameters and were trimmed to the minimal shared length. For the multilocus sequence analysis, gene sequences were concatenated and aligned according to the alphabetical order of the genes, ending in a sequence of 3095 bp (bp 1 to 768 for *16S rDNA*, 769 to 1411 for *acn*, 1412 to 1938 for *cts*, 1939 to 2591 for *pgi*, and 2592 to 3095 for *rpoD*).

Finally, for genetic relationship analyses, neighbor-joining (NJ) trees were generated in MEGA X software by using the Tamura–Nei evolutionary model with gamma correction and 1000 bootstrap replicates for all sequences [43,47–49].

2.5. Copper Tolerance

To evaluate whether the *Pseudomonas* strains isolated from Chilean fields presented a copper-tolerant phenotype, the minimum inhibitory concentration (MIC) of copper was found for each strain [28,31,50]. Briefly, different bacterial isolates were cultured in liquid medium CYEG (Casitone 1.7%, yeast extract 0.35%, and glycerol 2%) supplemented with increasing concentrations of copper sulfate (considering 1 mM $CuSO_4$ x $5H_2O$ = 63.5 µg/mL Cu^{2+}). The copper concentrations measured were 0, 8, 16, 32, 64, 80, 100, 160, and 200 µg/mL Cu^{2+}, using an incubation temperature of 28 °C. Each isolate was analyzed in triplicate, and bacterial growth was determined by spectrophotometry, measuring the optical density of the culture at 600 nm wavelength (OD_{600}) after 0, 24, 48, and 72 h of incubation. Each assay was started (0 h) with a fresh inoculum of the tested bacterial isolate, fixing an initial OD600 at 0.1. MIC was defined as the point where the OD_{600} of the bacterial culture at 24, 48, and 72 h was equal to (indicating no growth) or less (indicating possible cell death or lysis) tan OD_{600} at 0 h.

Pseudomonas isolates were considered copper tolerant when the minimum inhibitory concentration (MIC) was equal to or greater than 64 µg/mL [51].

2.6. Streptomycin Susceptibility

The minimum inhibitory concentration (MIC) of streptomycin was found based on the method described by Valenzuela et al. (2019) [52]. Briefly, bacterial isolates were grown overnight in LB broth (Luria-Bertani; 1% tryptone, 0.5% yeast extract, and 1% NaCl) at 28 °C, with constant agitation. Optical density was measured in a spectrophotometer, as described above, and all cultures were diluted in LB broth to adjust the OD_{600} to 0.3. Subsequently, 10 µL of each culture was plated onto CYEG agar (Casitone 1.7%, yeast extract 0.35%, glycerol 2%, and agar 12%) supplemented with streptomycin sulfate (Phytotechnology Labs) at concentrations of 0, 2, 4, 6, 8, 10, 12, 15, 20, 50, 100, 250, and 500 µg/mL. Three replicates were conducted for each concentration. Inoculated plates were incubated at 28 °C for 48–72 h. Experiments were performed in duplicate. The MIC of streptomycin was defined as the lowest concentration of streptomycin sulfate at which bacterial growth was inhibited after 48 h of incubation at 28 °C. *Pseudomonas* isolates with MICs $\geq$ 50 µg/mL were considered streptomycin resistant [28,52,53].

2.7. Antimicrobial Susceptibility Test (AST)

The in vitro susceptibility of bacterial isolates to five antibiotics used in human medicine was evaluated by using the disc diffusion method. Commercial discs for antibiogram (Thermo Scientific™, Waltham, MA USA, Oxoid™, Basingstokec, UK) with rifampin (5 µg), ampicillin (10 µg), gentamicin (10 µg), chloramphenicol (30 µg), and tetracycline (30 µg) were used according to the manufacturer's recommendations. Briefly, a fresh inoculum of each strain was diluted in a sterile saline solution (0.85% NaCl), and the OD_{600} was adjusted to 0.08–0.1 (equivalent to 0.5 on the McFarland turbidity scale). The medium chosen for the test was Mueller–Hinton agar (Thermo Scientific™ Oxoid™) suitable for AST, and the agar plates were poured on leveled surfaces to ensure a uniform depth of agar (~4 mm in depth). Using a sterile technique, the bacteria were inoculated into the Petri dish by spreading a homogeneous layer over the entire surface of the plate. Five antibiogram disks were applied over the inoculated agar, and plates were incubated for 16–18 h at 30 °C. At the end of the culture, the diameters of the zones of inhibition were measured to the nearest whole millimeter, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [54]. *E. coli* ATCC 25922 was used for quality control. Each experiment was repeated twice. The sensitivity of the bacteria to antibiotics was determined according to the specifications of the Clinical and Laboratory Standards Institute (CLSI) [54–57] and was defined as follows: susceptible (S), a status in which the growth of the bacteria is inhibited in vitro by the applied concentration of the antibiotic and the antibiotic dose is therapeutically effective; moderately susceptible (MS), bacterial response to the drug is lower, and the drug may fail to achieve a therapeutic response; or resistant (R), bacteria are not responsive to the given antibiotic, thus clearly indicating therapeutic failure.

For representation of antimicrobial susceptibility of all studied strains, a heat map was generated by using R software (https://www.R-project.org/) (accessed on 15 November 2021) (R Core Team, 2008), the "heatmap" package stats 3.6.0, and plotted by using the "ggplot2" package.

3. Results

3.1. Isolation and Identification of Pseudomonas from Orchards

The first choice of the bacterial isolates obtained was made based on the macroscopic characteristics of the colonies, such as color, shape, shine, and mucosity. Using these criteria, 298 isolates were obtained. Bacterial colonies that presented characteristics such as the classical morphology of *Pseudomonads* in KB medium (mucoid, spherical/convex colonies with a defined smooth border, shiny surface, and a fluorescent yellow color)

were selected for identification by 16S rRNA sequencing. For sixty-four isolates, the greatest identity matched with representatives of the genus *Pseudomonas* (Table 4). Among them, 19 isolates were most similar to *P. syringae*, 7 to *P. koreensis*, and 6 to *P. viridiflava*. Additionally, *P. gessardii*, *P. azotoformans*, *P. fluorescens*, and *P. marginalis* appeared in the first matches of a few isolates. Sequences of 17 isolates matched with at least two sequences among the following species: *P. fluorescens*, *P. koreensis*, *P. paralactis*, *P. putida*, *P. gessardii*, *P. reinekei*, *P. brenneri*, *P. punonensis*, *P. argentinensis,* and unidentified *Pseudomonas* sp. (Table 4). These 64 isolates identified as *Pseudomonas* spp. (Tables 1 and 4) were selected for further analysis.

Table 4. Bacterial species most closely related to *Pseudomonas* spp. isolates based on identity of 16S rRNA gene sequences.

	Isolates	Species Identification	Identity (%)	GenBank Accession Number [1]
Isolates matching with single *Pseudomonas* species	p22, p23, p24, p26, p27, p28, pJV.8r, pJ.1, pJ3.2, pJ5.1, pJ5.2, pH6.5, pH6.6, pH7.9, pH7.10, pH7.13, pH7.14, pH7.17, pH7.18	*Pseudomonas syringae*	99.5–100	MW138064
	pAZ.24, pAZ.25, pAZ.26, pH6.2, pFL4, pFL5	*Pseudomonas viridiflava*	100	OK091003
	p27Ch, p29Ch, p42Ch, pLO4.2, pLO5.2, p1.12, p2.2	*Pseudomonas koreensis*	99.9–100	MZ707723
	pJ6.3, p10.3, pLO2.3	*Pseudomonas azotoformans*	100	MW221357
	pA2.4, pA2.5, pA2.6	*Pseudomonas gessardii*	100	MT889683
	pHm27	*Pseudomonas putida*	100	MZ209185
	pJV.9r	*Pseudomonas marginalis*	100	OK086045
	pHm28	*Pseudomonas fluorescens*	99.3	KJ590506
Isolates matching with at least two *Pseudomonas* species	pFL2, pFL3, pFL13, pFL17, pJ4.2, pl1.3, pAII.1, pAII.2	*Pseudomonas paralactis*	100	MZ674188
		Pseudomonas putida	100	MZ497031
		Pseudomonas gessardii	100	MZ452411
	pFL8, p4.8, p9.7, p9.9	*Pseudomonas fluorescens*	100	MZ503692
		Pseudomonas koreensis	100	MW524107
	pAI.1, pT11	*Pseudomonas putida*	99.9	OK083428
		Pseudomonas koreensis	99.9	MZ965051
		Pseudomonas fluorescens	99.9	MZ914652
	pJS5.5	*Pseudomonas punonensis*	99.9	NR_109583
		Pseudomonas argentinensis	99.9	MZ853950
		Pseudomonas sp.	99.9	MW033798
	pl1.1	*Pseudomonas* sp.	100	MT354167
		Pseudomonas koreensis	100	MK790616
	pJS4.2	*Pseudomonas brenneri*	100	MZ914419
		Pseudomonas fluorescens	100	MW295497
Isolates matching with undefined *Pseudomonas* species	pl3.12	*Pseudomonas* sp.	100	MZ825296
	pl1.2	*Pseudomonas* sp.	100	MZ758888
	p4.3	*Pseudomonas* sp.	100	MW930799
	pAg2	*Pseudomonas* sp.	100	JN899567
	pJ4.1	*Pseudomonas* sp.	99.1	MW089209

[1] Accession number of the first match is reported. More than one sequence is only specified in cases where different species were found in the first positions with the same identity percentage.

3.2. Genetic Diversity of *Pseudomonas* spp. Isolates

Reference *Pseudomonas* species were selected according to the results of the 16S rDNA sequence analysis (Table 4). To study the genetic relationships among the selected strains, NJ trees were constructed based on the concatenation of all five genes (Figure 2) or single genes (Supplementary Figures S1–S5).

Figure 2. Neighbor-joining tree of the MLSA dataset. The total length of the compared sequences is 3095 bp in the final dataset. The seven major groups discussed in the text are labeled (A–G). Bootstrap scores greater than 80 are given at each node. *E. coli* K-12 (genome accession number U00096) strain was used as outgroup. The evolutionary distances were computed by using the Tamura–Nei method and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 2). The location of the isolates is represented with different markers next to each strain name as follow: empty square, Limache (Valparaíso); filled square, Hijuelas (Valparaíso); empty diamond, Chimbarongo (O´Higgins); filled diamond, Quillota (Valparaíso); empty triangle, Quillón (Bío Bío); filled triangle, Olmué (Valparaíso); filled inverted triangle, Teno (Maule); filled circle, Chillán (Bío Bío). Representative sequences from the genus *Pseudomonas* included as references are listed below, along with their geographic origins in parenthesis and accession numbers in square brackets: *P. azotoformans* LMG21611 (Japan) [LT629702], *P. fluorescens* FDAARGOS 1088 (Germany) [NZ_CP068151.1], *P. gessardii* BS2982 (France) [FNKR01000003], *P. koreensis* BS3658 (Republic of Korea) [LT629687.1], *P. putida* KT2440 (Japan) [NC_002947.4], *P. syringae pv. syringae* B728a (USA) [NC_007005.1], *P. syrigae pv. tomato* DC3000 (United Kingdom) [NC_004578.1] and T1 (Canada) [NZ_ABSM00000000], and *P. viridiflava* CFBP 1590 (France) [NZ_LT855380.1]. Abbreviations: *P. azotoformans* (P. azo), *P. fluorescens* (P. flu), *P. gessardii* (P. gess), *P. koreensis* (P. kor), *P. putida* (P. put), *P. syringae pv. syringae* (Pss), and *P. syrigae pv. tomato* (Pst). *P. viridiflava* (P. vir).

Figure 2 shows that 57 strains were grouped into seven genetic lineages (Figure 2, groups A–G), with identity ranging between 95.2 and 100% inside each group. The other seven isolates (pl3.12, pJS5.5, pJV.9r, pl1.2, pJ4.2, pFL5, and pFL4) had identity values lower than 95% (cutoff) when compared with the strains included in the seven groups. Thus, these isolates were set aside and were not included as part of a particular group. The identity among the 64 isolates ranged from 82.2 to 100%. The lowest identity percentage (82.2%) was seen when comparing sequences from the pJ4.2 and pJS5.5 strains. Groups A, B, and C were genetically closer to the reference sequences of *P. syringae* pv. *tomato, P. syringae* pv. *syringae*, and *P. viridiflava*, respectively, which all belong to the P. *syringae* complex [48]. Groups D, E, F, and G were closely related to the reference sequences of *P. koreensis*, *P. gessardii*, *P. azotoformans*, and *P. fluorescens*, respectively.

Group A was composed of 10 isolates that were retrieved from the Valparaíso region. Isolates from various locations (Quillota, Hijuelas, and Limache) clustered together, with identities ranging from 99.1 to 100%. Group B contained seven isolates from Quillón (Bío Bío region) grouped with the *P. syringae* pv. *syringae* B728a reference strain. In this group, identity percentages ranged from 98.9 to 100%, with pH6.5 and pH7.9 being the most dissimilar isolates.

Four isolates from the Maule and Bío Bío regions clustered with the *P. viridiflava* CFBP1590 reference strain to form group C, with identity percentages from 98.7 to 100%. Group D, one of the largest groups (along with group G), consisted of 15 isolates from the Valparaíso, O'Higgins, and Bío Bío regions grouped with the *P. koreensis* BS3658 reference strain. The identity percentage varied from 95.2 to 100%. Strains isolated from geographically distant points were closely related, as was the case for p2.2 and p27Ch (99.7% identical) isolated from Hijuelas and Chillan locations, respectively. Group E included four isolates from the Valparaíso region and the *P. gessardii* BS2982 reference strain. Inside this group, identity percentages ranged from 95.8 to 99.7%. Group F was the smallest, including only two isolates (from the same location, Quillón, Bío Bío region) whose sequences were 99.7% identical and clustered with the *P. azotoformans* reference strain. Finally, group G included 15 isolates and showed identity percentages ranging from 95 to 99.9%. This group included strains from six different orchards distributed in the Valparaíso, O´Higgins, Maule, and Bío Bío regions. Strains in this group were closely related to the *P. fluorescens* FDAARGOS 1088 reference strain.

Seven isolates could not be clustered in the NJ tree, namely pl1.2, pJ4.2, pFL4, pFL5, pJV.9r, pJS5.5, and pl3.12. For isolate pl1.2, the maximum identity was with pFL8 (95.3%), but with the rest of group D, the identity values were lower than 95%. A similar situation occurred with pJ4.2, pFL4, and pFL5 isolates, which were genetically closer to strains in group C with maximum identity values of 93.8% and 94.3%, when comparing pJ4.2 with pAZ.24/pAZ.25/pAZ.25 and pFL4/pFL5 with all the group C isolates, respectively. Isolate pJV.9r was more closely related to isolates belonging to groups F and G, with identity percentages of 95.2% and 95% when compared with pj5.1 (group G) and pHm28 (group F), respectively. Interestingly, isolates pJS5.5 and pl3.12 were not genetically closer to any of the formed groups, with maximum identity values of 86.2 to 87%, respectively, when compared with the rest of the isolates. This is consistent with their 16S rDNA sequences, which showed identity with other *Pseudomonas* species not being used as references in the MLSA analysis.

When individual genes were used to compare the isolates (Supplementary Figures S1–S5), similar clustering was seen in the respective dendrograms. The most conserved gene was 16S rDNA, with identity values over 95.4% (Supplementary Figure S1). The compositions of groups A, B, C, and D were conserved in all the constructed dendrograms, with the exception of the 16S rDNA-based analysis, where the major cluster included MLSA groups A and B, which were indistinguishable. Greater sequence variability was seen in the *pgi and rpoD* genes, mainly among the strains of groups D and G. In the NJ tree based on the *cts* gene (Supplementary Figure S3), only five groups were clearly distinguished. In this case, MLSA groups E, F, and G (Figure 2) clustered together, except for the pA2.4 and pA2.6

isolates, which were not grouped with the others. Additionally, the position of isolates pl3.12 and pJS5.5 in the tree differed with respect to the MLSA analysis. Furthermore, different results were seen for isolates pl1.2, pJ4.2, pFL4, pFL5, pJV.9r, pJS5.5, and pl3.12 (which were excluded from groups A–G based on the MLSA analysis). Meanwhile, pJS5.5 and pl3.12 were consistently excluded from the clusters; however, this was not the case for pl1.2, pJ4.2, pFL4, pFL5, and pJV.9r. In the *cts*- and *pgi*-based analyses, strain pl1.2 was included in group D with the reference strain *P. koorensis* BS3658. Isolate pJ4.2 was included in group C (including the *P. viridiflava* CFBP1590 reference strain) when analyses were based on either the *pgi* or *rpoD* genes. Otherwise, only pFL4 and pFL5 were included in group C in the analysis based on the *cts* gene. Isolate pJV.9r showed variable clustering depending on the gene analyzed; for example, in the *acn*-based tree, this strain was included in group E, but in the NJ tree based on the *rpoD* gene, this strain was more closely related to the strains of group F. Thus, pJV.9r did not show a clear genetic filiation with the other analyzed isolates. Finally, it should be noted that clustering based only on the *rpoD* gene best reflected the genetic relationship obtained through the analysis based on the five genes together (MLSA) (Figure 2 and Supplementary Figure S5).

3.3. Phenotypic Tolerance to Copper and Antibiotics

Since copper-based antimicrobials, together with the antibiotic streptomycin, have been the main bacterial control strategy for decades in agricultural crops [11,16,53], the susceptibility of the 64 isolates to these compounds was first evaluated. For this, the minimum inhibitory concentration (MIC) for each isolate was determined in the presence of variable concentrations of copper (Cu) or streptomycin (str). The details of the MIC obtained in each case are shown in Table 5. The results indicated that 70.1% of the isolates showed tolerance to copper, with MIC values greater than 64 µg/mL, and 45.3% were resistant to streptomycin (MIC values $\geq$ 50 µg/mL). Of the 29 isolates considered resistant to streptomycin, 16 had a highly resistant phenotype with MIC values above 200 µg/mL [52]. Among the 45 copper-tolerant isolates, 24 were also resistant to streptomycin (37.5% of the total). Resistant isolates to one or both antimicrobials typically used in agriculture (copper and streptomycin) were retrieved from all sampled locations (Figure 3). Only 14 isolates were susceptible to both copper and streptomycin, with representatives from the Valparaíso, Maule, O'Higgins, and Bío Bío regions (Table 5).

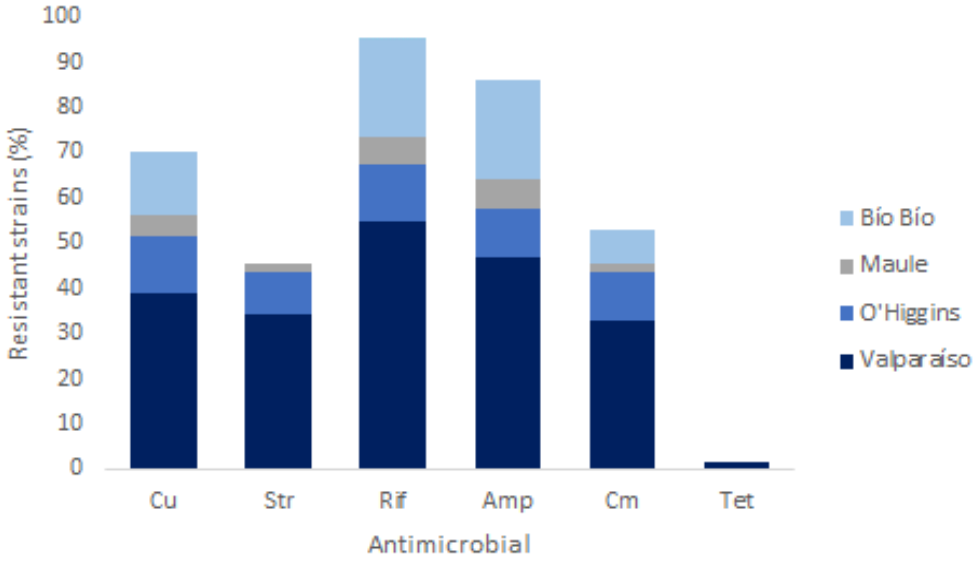

Figure 3. Presence and distribution of antimicrobial resistant *Pseudomonas* isolates from different Chilean regions. Data are expressed as percentage of total isolates. Antimicrobial are abbreviated as follows: Cu, copper; Str, streptomycin; Rif, rifampin; Amp, ampicillin; Cm, chloramphenicol; Tet, tetracycline.

Table 5. Antimicrobial susceptibility of the *Pseudomonas* isolates.

	MIC (μg/mL)		AST Test [1]				
Isolate	**Cu**	**Streptomycin**	**Ampicillin**	**Chloramphenicol**	**Gentamicin**	**Tetracycline**	**Rifampin**
pA2.4	64	15	R	R	S	S	R
pA2.5	32	4	R	S	MS	S	R
pA2.6	64	250	MS	S	S	S	R
pAI.1	32	6	S	S	S	S	R
pAII.1	32	100	R	R	S	S	R
pAII.2	64	50	R	R	S	S	R
pFL13	32	100	R	R	S	S	R
pFL17	64	250	R	R	S	S	R
pFL2	100	500	R	R	S	S	R
pFL3	80	500	R	R	S	S	R
pFL4	32	4	R	S	S	S	R
pFL5	32	4	R	MS	S	S	R
pFL8	32	15	R	R	S	MS	R
pJV.8r	32	100	MS	S	S	MS	R
pJV.9r	100	250	R	MS	S	R	R
pJ.1	64	50	S	MS	S	S	R
pJ3.2	64	15	R	R	S	S	R
pJ4.1	64	50	R	R	S	S	R
pJ5.1	32	100	R	R	S	S	R
pJ5.2	64	250	R	S	S	S	R
pJ4.2	100	250	R	R	S	S	R
pJ6.3	80	100	R	R	S	S	R
p1.12	80	12	R	R	S	S	MS
p10.3	64	250	R	R	S	S	R
p2.2	80	15	R	R	S	S	R
p4.1	32	12	R	R	S	S	R
p4.8	64	15	R	R	S	S	R
p9.7	32	15	R	R	S	MS	R
p9.9	32	15	R	R	S	S	R
p4.3	64	4	R	S	S	S	MS
pAZ.24	64	4	R	MS	S	S	R
pAZ.25	32	2	R	MS	S	S	R
pAZ.26	64	15	R	MS	S	S	R
p27Ch	32	15	R	R	S	S	R
p29Ch	32	15	R	R	S	S	R
p42Ch	64	15	R	R	S	MS	R
pH7.13	64	15	R	S	S	S	R
pH7.17	100	2	R	S	S	S	R
pH6.6	32	4	R	MS	S	S	R
pH7.10	80	6	R	S	S	S	R
pHm27	100	15	R	R	S	S	R
pH7.14	100	2	R	S	S	S	R
pHm28	80	15	R	R	S	S	R
pH7.18	100	4	R	S	S	S	R
pH7.9	80	4	R	S	S	S	R
pH6.2	32	2	R	S	S	S	R
pH6.5	32	10	R	S	S	S	R
p22	64	250	MS	S	S	S	R
p23	64	250	R	S	S	S	R
p24	32	500	R	S	S	S	R
p26	64	250	MS	S	S	S	R
p27	64	250	MS	S	S	S	R
p28	64	250	R	S	S	S	R
pT11	80	500	R	R	S	MS	R
pLO2.3	80	100	R	R	S	S	R
pLO4.2	100	100	R	R	S	S	R
pLO5.2	64	20	R	R	S	S	R
pl1.1	100	500	R	R	S	S	R
pl3.12	64	2	MS	S	S	S	R
pl1.2	64	100	R	R	S	S	R
pl1.3	64	100	R	R	S	S	R
pJS4.2	64	20	R	R	S	S	R
pJS5.5	64	15	MS	S	S	S	S
pAg2	64	100	R	R	S	S	R

[1] Susceptibility to antimicrobials not used in agriculture was determined by the disc diffusion method. S, susceptible; MS, moderately susceptible; R, resistant

In addition to streptomycin, we wanted to assess whether the isolates showed resistance to other antibiotics used in human or animal medicine. This could represent a significant health risk, considering the possible transfer of genetic resistance determinants between bacterial isolates, including those pathogenic to humans or animals. For this, an AST using the disc diffusion method was conducted with the antibiotics ampicillin (amp), rifampicin (rif), gentamicin (gn), chloramphenicol (cm), and tetracycline (tet). The results showed that 95%, 86%, 53%, and 1.6% of the isolates could be considered resistant to

rifampin, ampicillin, chloramphenicol, and tetracycline, respectively, with no isolates being resistant to gentamicin (Table 5 and Figure 3). Some isolates were considered moderately susceptible (MS), representing a few cases (one to seven) for each antibiotic (Table 5). Resistance to five of the seven antimicrobials evaluated was present in all regions of isolation, except for tetracycline, with only one resistant isolate isolated from the Valparaíso region, and for streptomycin, with no resistant isolates from the Bío Bío region (Figure 3).

To facilitate the analysis, bacterial isolates were grouped in a heatmap according to their antimicrobial resistance (Figure 4). The isolation region and the group to which they belong, based on the MLSA analysis, are also displayed. Overall, the results show that 96.9% of the isolates are tolerant or resistant to a combination of at least two of the antimicrobials evaluated, with only two isolates being resistant to a single antimicrobial. This is the case for the isolates pJS5.5 and AI.1, which showed resistance to copper and rifampin, respectively. Considering resistance characteristics, we found 17 phenotypes among the 64 isolates, ranging from resistance to a single antimicrobial to resistance to five of them (Figure 4). Among all the resistance phenotypes, multi-resistance to Cu-Str-Amp-Cm-Rif was the most frequent (23.4%), followed by the Cu-Amp-Cm-Rif and Cu-Amp-Rif resistance phenotypes, each representing 12.5% of the total isolates (Figure 4).

According to resistance phenotype, isolates were placed into four clusters (Clusters I–IV) (Figure 4). Cluster I contained twenty isolates, 75% resistant to rifampin and ampicillin, with 40% of isolates also being copper tolerant. Only two isolates (pJS5.5 and pAI.1) with resistance to a single antimicrobial (rif) were found in this group. In Cluster II, all the isolates were resistant to chloramphenicol and to ampicillin. Of the 34 isolates in this group, 52.9% were resistant to five antibiotics, and 44.1% were resistant to five antimicrobials (Cu + antibiotics). Isolate JV.9r constituted Cluster III, showing resistance to five antimicrobials (Cu, str, amp, rif, and tet), being the unique isolate resistant to tetracycline. Finally, Cluster IV was composed of three (33.3%) isolates sharing the resistance phenotype to streptomycin, rifampin, ampicillin, and copper. Among them, seven (77.8%) showed resistance to three antimicrobials (Cu, str, and rif). No isolates had resistance to chloramphenicol, gentamicin, and tetracycline.

From a genetic perspective, Cluster I contained all isolates from groups B and C classified according to MLSA. Cluster II contained a major portion of groups D and G, and Cluster IV contained mainly isolates from group A. Additionally, half of the isolates considered highly resistant to streptomycin were grouped in Cluster IV, all of which belonged to group A (MLSA), except for isolate pA2.6 (Figure 4).

All the studied isolates showed resistance to at least one of the tested antimicrobials, regardless of geographic location or sample type of origin, thus suggesting no relation between the spectrum of antimicrobial resistance and the geographic origin of the isolates (Figrues 3 and 4).

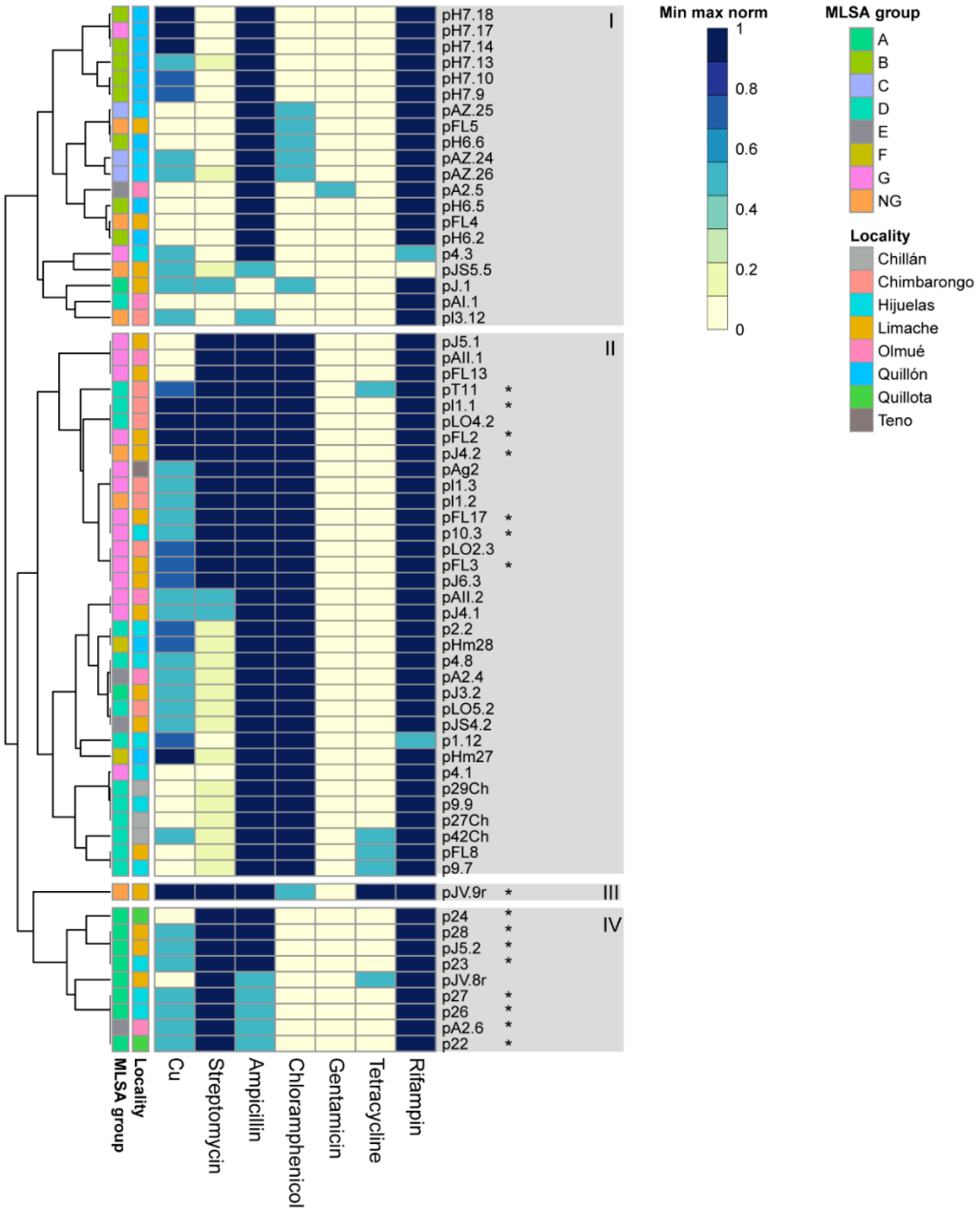

Figure 4. Heat map representation of *Pseudomonas* isolates antimicrobial susceptibility. The vertical axis contains the bacterial isolates which were clustered according to their susceptibility to the different antimicrobials (Clusters I–IV). In the case of Cu and streptomycin, both dark and light blue colored squares represent resistant phenotypes (with different MIC). For remaining antimicrobials, only dark blue represents resistant phenotype. Asterisks (*) highlight isolates classified as highly resistant to streptomycin (MIC $\geq$ 200 µg/mL). Isolation location and the genetic cluster to which they belong according to the MLSA analysis are also displayed.

4. Discussion

We analyzed the diversity of *Pseudomonas* species associated with tomato plants from Chilean orchards. *Pseudomonas* isolates were found in 92% of the orchards that were sampled (14 orchards in 8 locations) and were present in both healthy and diseased plants, representing 21.5% of the identified isolates. These results are consistent with previous studies, showing that the genus *Pseudomonas* has been consistently associated with tomato plants [3–5]. Indeed, Dong et al. (2019) reported that the genus *Pseudomonas* represents

36.76%, 25%, and 13% of the genera associated with the rhizosphere, root endophytes, and leaf endophytes of tomato plants, respectively [4].

Previous studies of *Pseudomonas* species associated with tomato plants in Chile have mainly focused on phytopathogenic *Pseudomonas*, such as *P. syringae, P. viridiflava*, *P. corrugate*, and *P. mediterranea*, as etiological agents of bacterial speck, stem necrosis, and pith necrosis of tomato [58]. In this study, phytopathogenic species were isolated from both healthy and diseased plants, consistent with the endophytic character of these phytopathogens; hence, isolates from five locations were identified as *P. syringae* pv. *tomato, P. syringae* pv. *syringae,* or *P. viridiflava* (Figure 2, MLSA groups A–C), all members of the *P. syringae* complex.

Members of the *P. fluorescens* group, such as *P. fluorescens, P. koreensis*, *P. gessardii*, and *P. azotoformans*, usually described as plant growth-promoting rhizobacteria (PGPR), were also obtained, with *P. fluorescens* and *P. koreensis* present in seven of the eight sampled locations. Different authors have described these species as being associated with plants or agricultural environments [59–61], so we expected to find them in association with tomato crops in Chile, but the distribution or proportion of the varied species found was previously unknown.

Although *P. fluorescens* has been studied primarily for its biostimulant properties and its potential as a biocontroller [5,62], its ability to emerge as an opportunistic pathogen has been reported in recent years [9], so its presence in tomato plants with symptoms of disease, as is the case of pAII.1, pAII.2, pJ5.1, pJ4.1, and pJ6.3 isolates (Figure 2, group G), must be carefully analyzed. Our identification of isolates as *P. koreensis* (Figure 2, group D) is similar, since some were obtained from plants with symptoms of illness. However, to our knowledge, there are no reports of *P. koreensis* with pathogenic characteristics [61].

Earlier MLSAs of *Pseudomonas* isolates, using the same or some of the genes analyzed in this study, have shown variable results regarding the resolution at the species level or the usefulness of individual genes in predicting genetic parentage. However, in this work, the use of five genes together allowed a greater resolution of the clustering of isolates in the NJ analysis, where each group was mainly associated with a reference isolate representative of a particular species. The same was not seen when analyzing the 16S gene by itself, where the high similarity between all the isolates studied did not allow adequate resolution, even though the genetic region used contained the gene fragment suggested by Singh et al., in studying the phylogenetic relationship of *Pseudomonas* species [46]. For example, in analyzing this gene, it was found that the isolates associated with the species *P. azotoformans*, *P. gessardii,* and *P. fluorescens* could not be clearly distinguished from each other. This would be expected since these species are defined as being closely related, even being grouped together in the past within the so-called "*P. fluorescens* group" [45]. The analysis of the individual genes showed that the result obtained from the individual analysis of the *rpoD* gene was the one that best represented the clustering results obtained by MLSA. This agrees with what was seen by Oueslati et al., who used the *rpoD*, *16S*, and *gyrB* genes to study the phylogenetic relationship of *Pseudomonas syringae* isolates [63]. Other authors have proposed that the *cts* gene alone can accurately predict the phylogenetic grouping for the *P. syringae* group [47]. However, in this study, the results of the analysis based only on the *cts* gene were the most dissimilar from those of the MLSA. This may be because the isolates we studied included diverse species, not only *P. syringae.* In this regard, the most conserved groups both in the MLSA and analysis of individual genes were those that were genetically related to *Pseudomonas* species of the *syringae* complex (*syringae* and *viridiflava*), thus confirming that these markers are useful for the phylogenetic analysis of these species [43,47].

It is worth mentioning that there are no earlier studies that analyzed the diversity of *Pseudomonas* by using the five housekeeping genes used in this study on isolates of Chilean origin. Generally, previous studies were conducted primarily with isolates from Europe and North America [64], making these results the first to focus on *Pseudomonas* spp. associated with tomato crops in Chile. We believe it imperative to continue genetic analysis

in this region since important genetic differences have been reported among isolates from different geographical origins. For example, in the case of *P. syringae* pv. *tomato*, differences in type III effector genetic markers have been associated with host-range determination of different strains [64,65].

The intensive use of agrochemicals to control different *Pseudomonas* species has promoted the selection of resistant bacteria. Furthermore, isolates resistant and multiresistant to these treatments have been increasingly reported [11,28]. This study was the first associated with tomato crops to evaluate the phenotypic and tolerance to antimicrobial compounds in agricultural environments in Chile. Samples of the same orchard in different seasons or years were not taken, so reliable conclusions related to bacterial community changes in different seasons or how it is antimicrobial resistance would be affected depending on the season cannot be drawn and require future studies.

The results showed variable resistance phenotypes among the *Pseudomonas* isolates, but all 64 isolates showed resistance to at least one of the antimicrobials evaluated. In general, according to the MLSA, the results suggest no relationship between antimicrobial resistance and the genetic group to which the isolates belonged. The resistance of the studied isolates to copper and streptomycin is not surprising, since this characteristic is widely reported in different bacterial species associated with agricultural crops, including *P. syringae* and *P. fluorescens*, among others [11,14,17,28].

With ampicillin, the high percentage of resistant isolates has a precedent in the study conducted by Hwang et al. [28], where the author proved that resistance to this antibiotic seems to be an ancestral trait in the *P. syringae* group. Additionally, Armalyté et al. described a high frequency of ampicillin-resistant *Pseudomonas* isolated from agricultural soil [37]. Ampicillin, a beta-lactam antibiotic, is affected by β-lactamases. Among them, extended spectrum β-lactamases (ESBLs) are enzymes of Gram-negative bacteria conferring resistance against β-lactam antibiotics. ESBL producing Gram-negative bacteria have been reported worldwide, and resistance genes of the ESBL type are mostly plasmid associated and therefore can spread among different bacteria by horizontal transfer, leading to widespread resistance among different bacterial species [66]. In concordance, Igbinosa et al., detected the *bla*TEM antibiotic resistance gene (β-lactamase conferring ampicillin resistance) in 12.5% of *P. putida*, 57.14% of *P. fluorescens*, 100% of *P. aeruginosa*, and 40% in other *Pseudomonas* species isolated from environmental water samples [35].

The same has not been observed with the antibiotic rifampicin, for which contradictory results have been reported. In the study of Hwang et al., only 16 out of 60 *Pseudomonas* isolates were shown to be phenotypically resistant to rifampin [28], but a different result was observed by Igbinosa et al., where *Pseudomonas* isolates from two locations showed 100% resistance to rifampin [35]. Rifampin resistance is generally mediated by rifampin ADP-ribosyltransferases or by a mutation in the subunit of RNA polymerase, which has been considered unusual among environmental bacteria [37,67]. However, our results showed a high frequency of rifampin-resistant phenotypes among *Pseudomonas* isolates, similar to that observed by Igbinosa et al. [35], a scenario that is consistent with a generalized increase in resistance to different antibiotics in environmental bacteria worldwide.

In the context of antibiotic resistance, it has been determined that *P. aeruginosa*, which has been extensively studied due to its effect on human health, possesses intrinsic resistance to some of these compounds, including ampicillin, chloramphenicol, and tetracyclines [68]. However, apart from the high frequency of resistance to ampicillin, these "intrinsic" resistance characteristics have not been observed in environmental or agricultural crop-associated *Pseudomonas* isolates in this or in previous studies [28,63,69]. Although resistance to these antimicrobials cannot be considered an intrinsic characteristic, some *Pseudomonas* isolates with phenotypes resistant to ampicillin, chloramphenicol, and tetracycline have been found in this study and previously [28,37,63,69]. In addition to ampicillin resistance, a chloramphenicol resistance phenotype was also seen with high frequency among the *Pseudomonas* isolates. While ampicillin resistance is often associated with the presence of beta-lactamases, chloramphenicol resistance is most often due to the presence

of chloramphenicol acetyltransferases (CATs). However, in both cases, the participation of efflux pumps or membrane-associated transporters that could confer multidrug resistance has been described [36,37]. This is the case for resistance–nodulation–cell division (RND) superfamily exporters, which play a major role in drug expulsion in *P. aeruginosa* but have also been reported in several environmental *Pseudomonas* spp. [37]. In the case of tetracycline, resistance is most often due to the acquisition of new genes that code for energy-dependent efflux of tetracyclines, proteins that protect bacterial ribosomes from the action of tetracyclines, or enzymatically inactivate tetracyclines [36]. Despite a great diversity of genetic determinants conferring tetracycline resistance among several bacterial species, in this study, only one isolate showed resistance to this antibiotic.

Regarding gentamicin, the absence of resistance among the isolates studied agrees with earlier studies where it was seen that resistance to this antibiotic is not a widespread characteristic in the genus *Pseudomonas* [28,63]. This can be explained since, in contrast to streptomycin, gentamicin binds to multiple sites on ribosomes; thus, several mutations in bacterial chromosome would be needed to generate spontaneous mutants resistant to gentamicin [38]. However, *Pseudomonas* is considered among the most competent of bacteria regarding DNA uptake, imparting it a high possibility of picking up genes from the environment, which may explain the development of several new resistance genes [35]. Moreover, genetic determinants of antimicrobial resistance are often associated with plasmids, transposons, or gene cassettes, and also may include integrative and conjugative elements [11,18,36]. Additionally, antibiotic resistance may be a function of more than one gene or even a combination of genetic and environmental factors, and a resistant phenotype can sometimes be observed without detecting a specific genetic determinant [35].

Aside from resistance to a single antimicrobial agent, the most challenging public health problem is the increasing number of multiresistant bacteria. In this study, antimicrobial resistance analyses showed that 96.9% of the isolates were phenotypically resistant to a combination of at least two of the antimicrobials assessed. Among all the resistance phenotypes, multiresistance to Cu-Str-Amp-Cm-Rif was the most frequent (23.4%). Currently, there is growing concern about the co-selection of bacteria resistance to antibiotics and metals (such as copper) due to the excessive use of these compounds in agriculture [25,26]. It has been shown that the presence of high concentrations of metals favors the co-selection of resistance to antibiotics [26,70], generating co-resistance (presence of different determinants of resistance in the same genetic element) and cross-resistance, where the same genetic determinant confers resistance to antibiotics and metals [25]. In this context, bacterial efflux pumps can capture and extrude many structurally diverse antibiotics, in addition to nonantibiotic compounds, such as metal ions. In Gram-negative bacteria, the multidrug-resistant phenotype is largely conferred by resistance–nodulation–cell division superfamily (RND) efflux systems contributing to the intrinsic resistance of different *Pseudomonas* species [71]. Thus, this type of efflux pump probably plays a role in the multiple resistance of the *Pseudomonas* isolates studied, but more genomic and functional studies are needed to corroborate this hypothesis.

Faced with the imminent problem of antibiotic resistance, it is necessary to develop, implement, or reinforce alternative control measures. In this context, reinforcement of preventive control of bacteriosis would allow for us to reduce the use of bactericides. In the nursery, it is critical to use bacteria-free seeds and test seedlings before delivery to growers. At the producer level, it is opportune to consider carrying out crop rotation, the cleaning of greenhouse structures, the elimination of plant residues from previous production, the use of resistant varieties, the reduction of relative humidity inside the greenhouse, and use of ozonated or ultrafiltered water for irrigation as principal preventive management [72]. Moreover, the use of non-conventional control strategies could be implemented, such as biocontrol or phage therapy [73], to reduce the use of agrochemicals. Finally, it is considered of great importance to establish a program of antimicrobial resistance surveillance as a critical step within risk-assessment schemes and for detecting new trends and emerging threats [74].

Although copper or streptomycin resistance has been identified in several phytopathogenic bacteria [11,53], we are aware of few studies reporting resistance in environmental bacteria that are considered to be nonpathogenic either to both antimicrobials (Cu and Str) or in combination with antibiotics for nonagricultural use. The results of this study show that there are multiresistant environmental *Pseudomonas* in Chile, probably the result of intensive agrochemical use. Although the genetic basis of resistant phenotypes was not addressed in this study, the results establish a precedent for concern, where environmental bacteria frequently associated with agricultural crops can constitute a natural reservoir of resistance genes, not only for phytopathogens but also for human pathogens [30], and may further threaten public health and ecological security via horizontal gene transfer [11,19,70,75]. Since horizontal gene transfer (HGT) is one of the mechanisms that contributes the most to the spread of resistance determinants, recent studies demonstrating HGT events between endophytic and epiphytic bacteria associated with plants are of importance, considering that endophytic bacteria associated with edible plants could easily enter in contact with human associated bacteria trough the food-chain [76,77]. Thus, the presence of *Pseudomonas* isolates that are resistant to conventional bactericides and other antimicrobials makes these bacteria an emerging threat to the agriculture industry and to human health.

5. Conclusions

Numerous *Pseudomonas* spp. associated with tomato crops in Chile were found in this study. The isolated *Pseudomonas* spp. belonged to distinct species based on multilocus sequence analysis (MLSA) and showed diverse antimicrobial response patterns, with most of the isolates showing resistance to at least two of the tested antimicrobial agents. Antimicrobial resistance dissemination among bacterial populations is an increasing challenge worldwide. Antibiotic resistance in *Pseudomonas* isolates, mainly *P. syringae*, *P. viridiflava*, and *P. fluorescens* species, recovered from vegetal tissue is of particular concern because these *Pseudomonas* spp. are considered phytopathogens or opportunistic pathogens. Additionally, multiresistant environmental *Pseudomonas* could be a vehicle for the transmission of resistance genes to human pathogens. For all of these reasons, phytopathogenic bacteria should be at the center of antimicrobial resistance studies, and surveillance throughout the agricultural environment is needed to detect emerging multiresistant phenotypes.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae8080750/s1, Figure S1. Neighbor-Joining tree based on *16S rRNA* gene sequences data set. The length of the 692 compared sequences is 768 b. Bootstrap scores greater than 80 are given at each node. *E. coli* K-12 693 strain was used as an outgroup. The evolutionary distances were computed using the Tamura-Nei 694 method and are in the units of the number of base substitutions per site. The rate variation among 695 sites was modeled with a gamma distribution (shape parameter = 2), Figure S2. Neighbor-Joining tree based on *acn* gene rDNA sequences data set. The length of the compared 698 sequences is 642 b. Bootstrap scores greater than 80 are given at each node. *E. coli* K-12 strain was used as an 699 outgroup. The evolutionary distances were computed using the Tamura-Nei method and are in the units of the 700 number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution 701 (shape parameter = 2), Figure S3. Neighbor-Joining tree based on *cts* gene rDNA sequences data set. The length of the 704 compared sequences is 526 bp. Bootstrap scores greater than 80 are given at each node. *E. coli* K-12 705 strain was used as an outgroup. The evolutionary distances were computed using the Tamura-Nei 706 method and are in the units of the number of base substitutions per site. The rate variaon among 707 sites was modeled with a gamma distribution (shape parameter = 2), Figure S4. Neighbor-Joining tree based on *pgi* gene rDNA sequences data set. The length of the compared se- 710 quences is 652 b. Bootstrap scores greater than 80 are given at each node. *E. coli* K-12 strain was used as an 711 outgroup. The evolutionary distances were computed using the Tamura-Nei method and are in the units of the 712 number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution 713 (shape parameter = 2), Figure S5. Neighbor-Joining tree based on *rpoD* gene sequences data set. The length of the compared sequences 822 is 503 b. Bootstrap scores greater than 80 are given at each node. *E. coli* K-12 strain was used as an outgroup. 823 The evolutionary

distances were computed using the Tamura-Nei method and are in the units of the number 824 of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape 825 parameter = 2).

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