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# Pathogenicity and Virulence-Associated Factors of *Pseudomonas syringae* pv. *syringae* and [*P. amygdali* pv. *morsprunorum*] Strains From New Zealand Sweet Cherry (*Prunus avium*) Orchards

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## ABSTRACT

Previously genetically characterised strains *of Pseudomonas syringae.* pv. *syringae* (Pss), [*P. amygdali* pv. *morsprunorum*] (Pam, syn. *P. s.* pv. *morsprunorum* race 1) and *Pseudomonas* spp. from New Zealand were characterised for their pathogenicity and aggressiveness in plant tissue and associated virulence factors. Lesions on detached, Pss-inoculated immature fruit increased rapidly in size and, at 10 days post inoculation (dpi), had larger areas under the disease progress curve (AUDPC) than Pam-inoculated fruit (48.9 and 22.0, respectively). Detached leaves infiltrated with Pss-developed symptoms within 1 dpi and from 2 dpi for Pam. Necrosis from most Pss strains extended into the leaf veins by 7 dpi, while Pam strains' necrosis was confined to the inoculation site. On detached 1-year-old cherry shoots, *Pseudomonas* spp. strains exhibited the smallest mean lesion size (2.1–2.4 mm), whereas larger mean lesion sizes were observed with Pss strains (5.7–13.7 mm) and Pam strains (3.9–14.0 mm). A functional T3SS was inferred for Pss and Pam strains based on the hypersensitivity reactions observed on tobacco leaves and symptoms elicited on cherry tissue. Syringomycin production was prevalent (88%) among Pss strains. In contrast, only 1.4% of Pam strains produced coronatine. Most Pss strains (97.0%) were able to catalyse ice formation. The coexistence of strains with varying degrees of virulence and non-pathogenic strains suggests a complex ecological balance, where multiple factors, including genetic variation, virulence traits and environmental conditions, shape the population dynamics and disease outcomes.

## 1 | Introduction

The cherry industry is a key player in New Zealand's fruit-growing industry, known for producing premium-quality cherries for both local and international markets. With 83.3% of the total cherry orchards, Central Otago is the primary cherry production region in New Zealand and this can be attributed to its favourable growing conditions. Central Otago's climate is well suited for cherry cultivation, with cold winters and hot summers, and low rainfall during the fruit ripening period, which

[*P. amygdali* pv. *morsprunorum*] is a name that has been used in the literature as a synonym for *P. s.* pv. *morsprunorum* and is currently awaiting formal validation. We use [*P. amygdali* pv. *morsprunorum*] throughout the text.

reduces the risk of rain-induced fruit cracking. It is also the driest, coldest and hottest part of New Zealand. Frosts are common in Central Otago, which has the highest number of ground frosts (when the air temperature 2.5 cm above a clipped grass surface falls to −1°C or lower), and air frosts (when air temperature measured in a screen by a thermometer 1.3 m above the ground falls below 0°C), of all cherry-growing regions in New Zealand (Macara 2015). Despite Central Otago's conducive growing conditions, bacterial canker of cherry caused by *Pseudomonas* species remains a significant challenge to the cherry industry in New Zealand. The disease can produce plant losses of 20%–50% and sometimes leads to the removal of entire cherry blocks (Marroni et al. 2021).

*Pseudomonas* is a ubiquitous group of Gram-negative gammaproteobacteria that can live in numerous environments as saprophytes or parasites and can cause diseases in over 180 different hosts (Berge et al. 2014). Within this diverse genus, most plant pathogens are grouped into the *Pseudomonas syringae* species complex, which includes taxonomically closely related species such as *P. amygdali*, *P. cerasi* and *P. savastanoi* (Young 2010; Gomila et al. 2017; Ruinelli et al. 2019) and more than 60 pathovars, each targeting specific hosts (Gardan et al. 1999; Xin, Kvitko, and He 2018). The classification of strains within the *Pseudomonas* species complex has evolved from a primarily morphological and physiological approach to a more molecular and genomic approach. Gardan et al. (1999) used DNA–DNA hybridisation to distinguish nine genomospecies within the *Pseudomonas syringae* species complex. Sarkar and Guttman (2004), on the other hand, divided the complex into four major clades through multi-locus sequence typing (MLST). Parkinson et al. (2011) later described seven phylogroups (PG) based on the phylogeny of the *rpoD* housekeeping gene sequence. More recently, Berge et al. (2014) used MLST to categorise strains within the *Pseudomonas* syringae species complex into 13 PG and 23 subgroups, encompassing both strains from crops and those isolated from non-cropping areas.

With advances in taxonomy and genomic techniques, the relatedness of strains within the *P. syringae* species complex has become clearer, leading to the reclassification of certain strains into more accurate taxonomic groups. This reclassification helps in understanding their phylogenetic relationships and pathogenic characteristics better.

Within the different classification schemes (e.g. into PG), virulence factors (used by the pathogen to overcome the plant defence system) have been used to further characterise strains within the *P. syringae* species complex and to relate to the symptoms they induce in host tissue (Gilbert et al. 2010; Giovanardi et al. 2018; Parisi et al. 2019; Ruinelli et al. 2019). It is recognised that strains in the *P. syringae* species complex use three major virulence-associated systems during the establishment of host-pathogen interactions: secretion of effector proteins through the type III secretion system (T3SS); production of phytotoxins and ice nucleation activity (INA) (Kennelly et al. 2007). The T3SS is a protein delivery organelle found in gram-negative bacterial pathogens that injects Type III effectors (T3E) directly into the host cell cytoplasm to suppress pattern-triggered immunity (PTI) (Jin et al. 2003; Galán and Wolf-Watz 2006; Coburn, Sekirov, and Finlay 2007). Effectors can be directly or indirectly recognised (through plant immune receptors such as nucleotide-binding domain and leucine-rich repeat region (NRL)-containing proteins) by proteins encoded by resistance (*R*) genes triggering effector-triggered immunity (ETI) (Doughari 2015; Arnold and Preston 2019; Naveed et al. 2020). Successful ETI usually translates into disease resistance and programmed cell death (hypersensitive response) (Jones and Dangl 2006). If PTI is suppressed by one or more pathogen effectors, pathogen infection of the host occurs and in the absence of effective *R* genes, both PTI and ETI are overcome, ultimately leading to effector-triggered susceptibility (ETS) (Arnold and Preston 2019; Naveed et al. 2020). The T3SS is essential for pathogenicity and its disruption (e.g. by effector gene deletion) renders pathogenic *Pseudomonas* strains avirulent (Lindeberg, Cunnac, and Collmer 2009; Xin, Kvitko, and He 2018). The particular range of effectors produced by a specific *Pseudomonas* strain is thought to influence its host range or host specificity (Fouts et al. 2003).

Ice formed in, or on, frost-sensitive plants causes mechanical disruption of cell membranes, resulting in frost damage (Burke et al. 1976) and predisposing frost-sensitive plants to infection by *P. syringae* (Lindow 1983; Kennelly et al. 2007; Renick, Cogal, and Sundin 2008). This is because ice formation-related damage to plant tissues results in leakage of nutrients and water from host cells which some pathogens, including *P. syringae*, are able to hijack to facilitate their entry. Certain Gram-negative proteobacteria (e.g. *P. syringae, Xanthomonas* spp., *Erwinia* spp. and *Pantoea* spp.) exert ice nucleation activity. This property is the result of the production of a protein, InaZ, that can act as an ice nucleus, catalysing ice formation within plant tissues at temperatures just below 0°C (Lindow 1983). Ice nucleation active (Ice+) bacteria may promote frost injury in plants by impairing their supercooling ability.

In addition, some strains within the *P. syringae* species complex can produce phytotoxins to overcome the plant defence system. Unlike T3SS, phytotoxins are not essential for pathogenicity, but they may contribute to increased disease severity, spread *in planta* or habitat colonisation (Volksch and Weingart 1998; Bender, Alarcón-Chaidez, and Gross 1999). One example is the chlorosis-inducing toxin, coronatine, which is produced by strains of [*P. amygdali* pv. *morsprunorum*] (Pam, syn. *P. s.* pv. *morsprunurum* race 1); *P. syringae* strains produce syringomycin toxins. Coronatine mimics the plant hormone jasmonic acid, and through antagonism with the salicylic acid pathway, can inhibit its accumulation in stomatal guard cells, suppressing stomatal closure and facilitating *P. syringae* entry into the apoplast (Melotto, Underwood, and He 2008). Syringomycin is a necrosis-inducing toxin that induces pore formation on plant membranes, leading to the leaking of nutrients, and acts as a biosurfactant and induces increased wetness of the plant surface, which enhances bacterial movement (Xin, Kvitko, and He 2018).

The bacterial canker of cherry is caused by *P. syringae*: *P. s.* pv. *syringae* (Pss), Pam and *P. s.* pv. *morsprunorum* race 2 (Psm2). Recent studies using phylogeny based on the *gltA*

gene sequence and multi-locus sequence analysis (MLSA) investigated the diversity and prevalence of New Zealand *Pseudomonas* strains from cherry orchards in Central Otago. The study by Marroni et al. (2023), showed that within the *P. syringae* species complex, strains from Central Otago orchards belonged to two main taxonomic groups, Pss and Pam, in PG2 and PG3, respectively (Berge et al. 2014). Additionally, non-pathogenic strains classified as *Pseudomonas* spp. (as they aligned with other well-known, non-pathogenic *P.* spp. in the phylogenetic analysis) were also present. The results by Marroni et al. (2023) confirmed previous findings of Pss and Pam as major pathogens on cherries in New Zealand, and further highlighted the co-occurrence of non-pathogenic *P.* spp. and pathogenic Pss and Pam strains within the same orchard, as previously reported (Vicente et al. 2004; Marroni 2013; Visnovsky et al. 2019). The study also concluded that Pss was the most prevalent pathovar found across Central Otago orchards and tissue types (cankers, fruit lessons, bacterial leaf spots symptomatic/asymptomatic buds).

Genetic characterisation of plant pathogens and the identification of virulence-associated factors are complementary approaches that provide valuable insight into the pathogenicity of *Pseudomonas* spp. The aims of the present study were to investigate the virulence-associated factors, pathogenicity (i.e. the ability to infect a particular host species) and aggressiveness (which encompasses the severity of symptoms, rate of symptom development and ability to produce inoculum in host tissue) of Pss, Pam and *P.* spp. strains obtained from cherry orchards in Central Otago, New Zealand. Building upon the work of Marroni et al. (2023), we sought to gain a more comprehensive understanding of the mechanisms by which these strains cause disease in cherry trees.

## 2 | Materials and Methods

### 2.1 | Bacterial Strains

#### 2.1.1 | Strains and Culture Conditions

*Pseudomonas* strains were sourced from a collection of 250 strains from both symptomatic and asymptomatic tissue as outlined by Marroni et al. (2023). The strains were previously classified into 153 Pss in PG2, 70 Pam in PG3 and 27 *P.* spp., and the genetic characterisation was complemented by pathogenicity testing in immature cherry fruit (Table S1). In this study, further tests were conducted on a subset of strains representing the group's diversity or, in all 250 strains, as indicated in the specific sections below.

All bacterial strains were stored at −80°C in liquid Luria Bertani (LB) (Oxoid) media, supplemented with 30% glycerol and grown routinely on *Pseudomonas* F agar (Thermo Fisher Scientific). When required, bacterial suspensions were prepared by taking a 1-mL aliquot from a 16-h LB culture that had been maintained at 28°C. This aliquot was centrifuged at 6600 *g* for 5 min, the pellet resuspended in sterile 10 mM $MgSO_4$ and the concentration adjusted to $1 \times 10^6$ cells/mL or as indicated.

### 2.2 | Pathogenicity on Cherry Tissue

#### 2.2.1 | Progression and Severity of Symptoms on Detached Cherry Fruit

This test was conducted on a subset of 35 strains, selected from the original set of 250 strains, to represent the phylogenetic diversity (PG2, PG3 and *P.* spp.), tissue types (bud, leaf, fruit, canker and leaf scar) and orchards in the Central Otago region of New Zealand (Marroni et al. 2023). The subset included 12 Pss strains, 18 Pam strains and five *P.* spp., as described in Table 1. The progression and severity of symptoms were studied following inoculation of immature detached cherry fruit as described by Marroni et al. (2023). In brief, freshly picked immature sweet cherry ('Lapins') fruit collected from a commercial orchard in Central Otago were surface sterilised by immersion in 1% sodium hypochlorite for 1 min and rinsing three times in sterile distilled water (SDW). Bacterial suspensions containing approximately $10^6$ cfu/mL of each of the test strains were prepared. Immature cherry fruit were inoculated using sterile toothpicks that were dipped briefly (~1 s) into a suspension and then stabbed into the fruit cheek. Each individual fruit was stabbed at one site (three replicates) to a depth of ~2 mm. The fruit were each placed on a Petri dish lid or bottom that had been lined with sterile moist filter paper and the plates were set out in a 2-Latinised row-column design on a tray. The tray, which had also been lined with moist sterile paper towels, was covered with a plastic bag to maintain high humidity. The plates were incubated at 22°C under a 16 h:8 h light/dark cycle for 2–10 days. A known pathogenic strain of Pss, ICMP 21874 (Visnovsky et al. 2019) and a 10 mM $MgSO_4$ negative control were included. Lesion size (mm) was measured after 2–7 and 10 days post inoculation (dpi). Measurements were made in two perpendicular directions with a digital calliper. Two independent experiments were performed for the set of 35 strains. Results for lesion size were analysed by ANOVA using Genstat (Payne, Murray, and Baird 2019). Lesion severity over time was determined using the area under the disease progress curve (AUDPC) calculated using the equation:

$$\sum_{i=1}^{3} 0.5^{*}\left(\left(\mathrm{mLesion}_i - 1\right) + \left(\mathrm{mLesion}_{i-1} - 1\right)\right) * \left(\mathrm{dpi}_i - \mathrm{dpi}_{i-1}\right)$$

where $\mathrm{mLesion}_i$ was the mean lesion size for a single fruit at dpi *i*. $\mathrm{mLesion}_0$ is 1 and $\mathrm{dpi}_0$ is 0. The initial lesion size of 1 mm is subtracted from the mean lesion size.

#### 2.2.2 | Pathogenicity on Cherry Leaves

Pathogenicity on cherry leaves was studied with the method of Marroni et al. (2021). Newly emerged, fully expanded leaves (including the pedicel) were excised from the shoots of visually healthy 'Lapins' sweet cherry potted plants maintained at The New Zealand Institute for Plant and Food Research Ltd. (PFR) at Lincoln, New Zealand. These leaves were surface sterilized (immersion in 1% sodium hypochlorite for 1 min and rinsing three times in SDW) and dried in a laminar flow cabinet. Pathogenicity assays were conducted for the 35 strains,

**TABLE 1** | Characteristics of 35 strains representative of the *Pseudomonas* spp., *P. syringae* pv. *syringae* (Pss) or [*P. amygdali* pv. *morsprunorum*] (Pam) isolated from Central Otago (CO) cherry orchards in 2015.

| Isolate code | Taxonomic classification | Plant part | Geographical origin | Tobacco[a] | Syr[b] | Cor[c] | INA[d] |
|---|---|---|---|---|---|---|---|
| CO14 | *P.* sp. | Bud | Roxburgh | − | − | − | − |
| CO20 | Pam | Leaf spot | Cromwell | + | − | − | − |
| CO22 | Pam | Leaf scar | Cromwell | + | − | − | − |
| CO23 | Pss | Fruit | Cromwell | + | + | − | + |
| CO25 | Pss | Leaf spot | Cromwell | + | + | − | + |
| CO30 | Pam | Canker | Cromwell | + | − | − | − |
| CO34 | Pss | Leaf spot | Cromwell | + | + | − | + |
| CO44 | Pam | Leaf spot | Alexandra | + | − | − | − |
| CO48 | Pam | Leaf spot | Alexandra | + | − | − | − |
| CO55 | Pss | Leaf spot | Alexandra | + | + | − | + |
| CO61 | Pss | Bud | Cromwell | + | + | − | + |
| CO63 | *P.* sp. | Bud | Cromwell | − | − | − | − |
| CO80 | Pam | Leaf scar | Roxburgh | + | − | − | − |
| CO81 | Pam | Leaf scar | Roxburgh | + | − | − | − |
| CO86 | Pam | Leaf spot | Roxburgh | + | − | − | − |
| CO95 | Pss | Canker | Cromwell | + | + | − | + |
| CO114 | Pam | Canker | Cromwell | + | − | + | − |
| CO119 | Pss | Canker | Cromwell | + | + | − | + |
| CO150 | Pss | Leaf spot | Clyde | + | + | − | + |
| CO154 | Pss | Leaf spot | Clyde | + | + | − | + |
| CO161 | Pss | Leaf spot | Alexandra | + | − | − | − |
| CO163 | Pam | Canker | Alexandra | + | − | − | − |
| CO167 | Pam | Canker | Alexandra | + | − | − | − |
| CO173 | Pam | Leaf spot | Alexandra | + | − | − | − |
| CO179 | Pam | Leaf spot | Alexandra | + | − | − | − |
| CO180 | Pss | Canker | Alexandra | + | + | − | + |
| CO187 | Pam | Canker | Alexandra | + | − | − | − |
| CO189 | Pam | Leaf spot | Alexandra | + | − | − | − |
| CO199 | Pam | Bud | Cromwell | + | − | − | − |
| CO201 | Pam | Bud | Cromwell | + | − | − | − |
| CO231 | Pss | Canker | Alexandra | + | − | − | + |
| CO243 | *P.* sp. | Bud | Alexandra | − | − | − | − |
| CO251 | Pam | Canker | Cromwell | + | − | − | − |
| CO913 | *P.* sp. | Bud | Cromwell | − | − | − | − |
| CO929 | *P.* sp. | Bud | Clyde | − | − | − | − |

*Note:* The taxonomic affiliation presented in this table is based on the *gltA* and MLSA phylogenies and pathogenicity on detached immature cherry fruit as described by Marroni et al. (2023). + ice nucleating strain, +, positive result; −, negative result.
Abbreviations: Pam, [*Pseudomonas amygdali* pv. *morsprunorum*]; Pss, *Pseudomonas syringae* pv. *syringae*.
[a]Pathogenicity on tobacco leaves indicated by a hypersensitive reaction at 48 h after inoculation.
[b]Syringomycin production.
[c]Coronatine-encoding genes.
[d]Ice nucleation activity (INA).

selected as before (Table 1) by infiltrating 50–80 μL aliquots of bacterial suspensions ($1 \times 10^6$ cells/mL) into the abaxial leaf surface using a 1-mL syringe (no attached needle) and applying gentle pressure until the mesophyll became water soaked. Three leaves were infiltrated with up to three test strains, a positive control (strain of Pss ICMP 21874) and a negative control (10 mM $MgSO_4$). The inoculated leaves were each placed on a Petri dish containing water agar (10 g/L). Moist filter paper was used to cover the Petri dishes, which were also sealed with Parafilm. The Petri dishes were arranged in a 4-Latinised row-column design and left to incubate at 22°C for up to 14 days under natural daylight conditions. Daily observations allowed the time of the first symptom appearance (necrosis) to be recorded. Lesion size (mm) was measured at 7 and 14 dpi. Measurements were made in two perpendicular directions using an electronic calliper. Two separate experiments were performed for the set of 35 strains and the data for each assessment analysed separately by ANOVA using Genstat (Payne, Murray, and Baird 2019). AUDPC was not calculated for the leaves.

#### 2.2.3 | Pathogenicity on Cherry Shoots

This test was carried out for the same 35 strains described in Table 1. Shoots collected in July 2020 from a 'Lapins' sweet cherry commercial orchard that had been deemed healthy by visual inspection were used in this experiment. Dormant 1-year-old shoots of ~30 cm in length and 6 mm in diameter were surface sterilised (2-min immersion in 1% sodium hypochlorite followed by 5-min rinse under running tap water), left to dry on paper towels in a laminar flow cabinet and then divided into 15-cm segments. Detached twig inoculations were carried out according to Li et al. (2015) with some modifications. In brief, 20 μL of a bacterial suspension of ~$10^8$ cfu/mL was placed onto the cut end of each shoot from which the upper 5 mm had been removed. The suspension was allowed to absorb for 5–10 min, after which the shoot was covered with Parafilm. Approximately 5 mm was then removed from the basal ends of the shoots and the cut bases were immersed immediately in water-saturated Oasis (Oasis Floral Products). The shoots were arranged inside transparent plastic boxes in a 4-Latinised row-column design (2 rows × 19 columns per replicate) and incubated at 15°C for 1 week with a 16 h:8 h light/dark cycle. The shoots were then stored at −2°C in a freezer unit (Gallenkamp Cooled Incubator INF.631.Q with Lascar Electronics EL-GFX-DTC thermocouple sensors) for 1 week, also with a 16 h:8 h light/dark cycle, followed by storage at 15°C for 4 weeks under the same light/dark conditions. At this time, a scalpel was used to remove the bark so that the length (mm) of necrosis from the inoculated tip could be measured on two opposite sides of the twig using a calliper. The experiment was repeated twice. Data for each assessment were analysed separately by ANOVA using Genstat (Payne, Murray, and Baird 2019).

#### 2.2.4 | *Pseudomonas* Population Study on Detached Cherry Leaves

Lapins' sweet cherry detached leaves were used for *Pseudomonas* population growth studies on nine strains, including four Pss, three Pam strains and two *P.* spp. (Figure 5). The nine strains were chosen to represent the phylogenetic diversity (PG2, PG3 and *P.* spp.) and various tissue types (canker, leaf spot and necrotic fruit for Pss; leaf spot and canker for Pam) (Table 1). Reference strains, ICMP 21874 (Pss) and SBV PS6 (Pam) were positive controls and 10 mM $MgSO_4$ was used as a negative control.

The inoculation by infiltration method described previously Marroni et al. (2021) was applied to detached cherry leaves using a $10^5$ cfu/mL bacterial suspension (low inoculum concentration), resulting in approximately $10^4$ cfu/mL at the inoculation site. Each strain was infiltrated into nine replicate leaves (one isolate per leaf) and placed individually into Petri dishes containing water agar (Difco, 10 g/L), covered with moist filter paper and sealed with Parafilm. The leaves were placed in 'plots' of three plates, such that one plate per plot could be used at 0, 3 and 6 dpi. The plates were as arranged in a 2-Latinised row-column design (2 rows × 5 columns per replicate) and incubated at 22°C under a 16 h:8 h light/dark cycle. At each time point (0, 3 and 6 dpi), three leaves were selected per strain for analysis. Leaf discs of ~1 cm diameter were cut from the inoculation site using a sterilised Eppendorf tube. These discs were homogenised in 1 mL of 10 mM $MgSO_4$ and 100 μL of a serial dilution plated out onto *Pseudomonas* F plates. Bacterial cell concentration per mL was determined from the cfu value after 48 h of incubation. The experiment was conducted twice. Estimated cfu/mL of the original suspension and associated 95% confidence limits were obtained on the link (log) scale, setting the offset to 0. All analyses were carried out with Genstat (Payne, Murray, and Baird 2019).

#### 2.2.5 | Vascular Movement of Strains Inoculated Into Petioles of 'Lapins' Potted Plants

Petioles of fully expanded leaves located midway along a one-year-old twig of 'Lapins' sweet cherry potted plants were inoculated using seven strains. The same Pss, Pam and *P.* spp. as per the leaf growth experiment were used, except for CO23 (a strain of Pss). These strains still represented the phylogenetic diversity and various tissue types as in the leaf growth studies (Table 1). Only one Pss strain, ICMP 21874 was used as the positive control and 10 mM $MgSO_4$ as the negative control. A single drop (5-μL) of ~$10^6$ cfu/mL bacterial suspension of one of the selected strains (one strain per plant) or SDW was deposited at the puncture site on one leaf petiole per plant immediately following needle puncture. Clear plastic bags containing two moist paper towels were fastened at the base of the inoculated branches to maintain high humidity for 24 h after inoculation. The plants were placed in a 3-block-Latinised design in a glasshouse, with each block representing one replicate of nine treatments, and maintained at 22°C and 12 h:12 h light/dark conditions for 14 days. Following incubation, the branches were cut 5 cm above and below the petiole inoculation point and these samples surface sterilised in a laminar flow cabinet (20 s in 70% vol/vol ethanol followed by two rinses in SDW). To measure bacterial migration, each sample was dissected into seven segments: three above (apical, middle and base of the lamina) and three below the inoculation point (including the point of petiole attachment to the main branch, and 1-cm segments from above and below), along with

the segment coinciding with the inoculum point (Figure 6D). Using a sterilised Eppendorf tube (one new tube per sample), an approximately 1-cm diameter leaf disc was cut from the centre of each lamina section. A sterile scalpel was used to dissect the inoculated petiole and the segments taken from above and below the point of petiole attachment. The sections were placed in clear plastic bags labelled A–F to identify their point of origin. Samples were subsequently homogenised in 1 mL of 10 mM $MgSO_4$ using a hammer (three times) and a BIOREBA handheld homogeniser. A 100-μL sub-sample of was plated in triplicate on *Pseudomonas* F plates and incubated for 48 h at 27°C under natural daylight conditions, after which the presence/absence of *Pseudomonas*-like colonies was assessed, colony counting was performed and cfu/mL of the original suspension determined.

## 2.3 | Virulence-Associated Factors

### 2.3.1 | Presence of T3SS

Hypersensitivity Reaction (HR) tests were conducted on the leaves of glasshouse-grown, three-month-old tobacco (*Nicotiana glutinosa*) plants for the subset of 35 strains listed in Table 1. The assays involved infiltration of 50–100 μL aliquots of bacterial suspensions ($1 \times 10^6$ cells/mL) into the abaxial surface of fully expanded leaves using a 1-mL syringe (no attached needle) and applying gentle pressure until the mesophyll became water-soaked. Each leaf was treated with a positive control (strain Pss ICMP 21874), a negative control (10 mM $MgSO_4$) and up to four different test strains, with all inoculations performed on the same day. There were three replicates per strain set up in separate plants. Plants were arranged on trays in a random pattern and held at 20°C under a 16 h:8 h light/dark cycle in a glasshouse. Symptom expression indicative of a hypersensitive response was assessed 24 h post-inoculation. A positive hypersensitivity reaction observed on tobacco leaves indicates the presence of a functional T3SS (Jones and Dangl 2006).

### 2.3.2 | Production of Phytotoxins: Syringomycin and Coronatine

Production of syringomycin and coronatine was evaluated in all 250 strains (Marroni et al. 2023). A plate bioassay was carried out as described by Gross and DeVay (1977), with some modifications. Following point inoculation (in triplicate) onto potato dextrose agar (PDA; Difco) plates, *Pseudomonas* isolates were grown at 25°C under natural daylight conditions for 4 days. During the same period and growth conditions, a culture of the syringomycin-sensitive fungus, *Geotrichum candidum* (ICMP 17067), was grown on PDA. These fungal plates were washed with 10 mL of SDW, and the wash was filtered through two layers of sterile muslin cloth. A hand-held atomiser (Air Brush RA110 Remington) was used to spray the resulting spore suspension (concentration not determined) onto the surface of the 4-day-old bacterial cultures. Each plate was divided into four quadrants, and each quadrant was streaked with a different *Pseudomonas* test strain. The plates were then incubated for 2 days at 25°C under natural daylight conditions. Syringomycin production was detected by the presence of zones of fungal inhibition around the bacterial colonies.

The primers and PCR conditions developed by Bereswill et al. (1994) were used to amplify a 650-bp fragment indicative of the presence of the *cfl* gene encoding for coronatine syntheses. The PCR reaction was carried out in a total volume of 20 μL containing 1 μL of each primer (5 μM), 2 μL of 10× buffer (Invitrogen, Carlsbad, CA) 1 μL of $MgCl_2$ (25 mM), 2 μL of deoxynucleoside triphosphates (dNTPs; 2 mM), 0.10 μL of Taq polymerase (5 U/μL; Invitrogen) and 1 μL of template DNA (50–100 ng) in sterile water. A T100 Thermal Cycler (Bio-Rad Laboratories Inc.) was used for the PCR reaction. PCR products (10 μL) were separated on a 1.5% agarose gel and visualised using a MultiDoc-It Digital Imaging System (UVP/Bio-Strategy), with a molecular size marker (hyperladder 100 bp, Bioline) used for fragment size determination. Strain LMG2222, known to produce coronatine, was used as a positive control (Bultreys and Gheysen 1999).

### 2.3.3 | Ice Nucleation Activity

The INA of the collection of 250 strains was determined by the droplet-freezing method (Vali 1971). Five droplets of approximately $10^7$ cells/mL bacterial suspension (confirmed by dilution plating on KB) of each isolate were pipetted onto an aluminium boat coated with paraffin wax. These boats were floated for 5 min in a refrigerated (−5°C) bath containing a 50:50 mixture of ethylene glycol:water. The presence of frozen droplets indicated ice nucleation active (Ice+) strains. Sterile water, a Pss Ice+ (ICMP 21784) and an Ice− strain (SBV-PS6) were included in each boat as a negative and positive control, respectively.

# 3 | Results

## 3.1 | Pathogenicity in Plant Tissue

### 3.1.1 | Pathogenicity on Immature Cherry Fruit

At 2 dpi, *P.* spp. and the 10 mM $MgSO_4$ negative control caused browning limited to the inoculation site. In contrast, Pss and Pam strains produced lesions that extended from the point of inoculation (Figures 1 and 2). Except for isolate CO161, Pss strains produced distinctive black/necrotic lesions. Pam lesions had a water-soaked appearance. On fruit inoculated with Pss strains, the mean lesion size was 2.2 mm at 2 dpi, and this increased rapidly to 15.3 mm at 10 dpi. At 10 dpi, the lesions showed decay and oozing at the centre, indicating advanced disease progression (Figure 2). All Pss strains had a similar mean lesion size at each of the assessments, with the exception of strain CO161. The mean lesion size for CO161 was significantly ($p = 0.017$ at 2 dpi and $p < 0.001$ at 7 and 10 dpi) smaller (2.1 at 2 dpi and 5.94 mm at 10 dpi). The mean lesion size for Pam strains was significantly ($p < 0.001$) smaller than for Pss strains, increasing from 2.2 mm at 2 dpi to 4.7 mm at 10 dpi.

AUDPC values at 10 dpi revealed differences in symptom progression between fruit inoculated with Pss, Pam and *P.* spp.

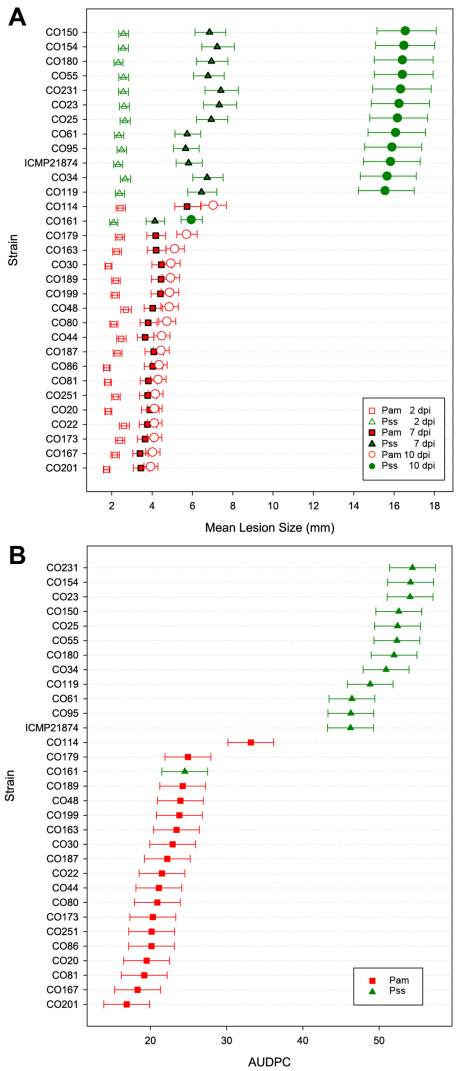


**FIGURE 1** | Aggressiveness of *Pseudomonas* strains from Central Otago (CO) (Marroni 2021). (A) Mean lesion size on detached immature 'Lapins' cherry fruit following wound inoculation with a bacterial suspension ($1 \times 10^6$ cells/mL) and incubated at 22°C of a subset of 35 strains including *P. syringae* pv. *syringae* (Pss), [*P. amygdali* pv. *morsprunorum*] (Pam), *P.* spp. and a negative control (10 mM $MgSO_4$), at 2 days post inoculation (dpi), 7 dpi, and 10 dpi. Isolates are in increasing order of the mean lesion size at 14 dpi. (B) Mean area under the disease progress curve (AUDPC) at 10 dpi. Note that the mean for the Control and *P.* spp. strains after discounting the 1-mm lesion corresponding to the inoculation puncture was 0, and therefore is not shown. Error bars represent 95% confidence limits.

strains (Figure 1). Mean AUDPC was significantly lower for Pam strains than for Pss strains (22.0 vs. 48.9 mm; $p < 0.001$). Lesions generated by the negative control and *P.* spp. strains were restricted to the inoculation site, with an AUDPC of 0.0, which was significantly smaller than for Pss and Pam strains ($p < 0.001$ in both comparisons). Strain CO161, which was classified as Pss, produced lesions that had a similar appearance and progression to lesions produced by Pam strains. Overall, 10 dpi data from inoculated immature cherry fruit distinguished Pss and Pam from non-pathogenic *P.* spp., and, with the exception of CO161, also distinguished Pss from Pam strains.

### 3.1.2 | Pathogenicity on Cherry Leaves

Pss and Pam strains induced necrosis on cherry leaves, while no lesions were observed after infiltration with the negative control or *P.* spp. strains (Figure 3). On the infiltrated leaves, symptoms appeared within 1 dpi for Pss strains and from 2 dpi for Pam strains and CO161. At 7 dpi, the necrosis caused by most Pss strains had extended considerably from the inoculation site and into the leaf veins, with a maximum lesion size of 9.15 mm and an average of 6.2 mm. For all Pam strains, necrosis was limited to the inoculation site; the maximum lesion size reached 4.3 mm and the mean lesion size was 3.4 mm. Inoculation with *P.* spp. strains did not elicit any leaf symptoms by 7 dpi (Figure 3). The mean lesion size at 7 and 14 dpi was significantly ($p < 0.001$ both) larger for Pss strains (6.2 and 8.2 mm, respectively) than for Pam strains (3.8 and 4.3 mm, respectively). By 14 dpi, all Pss strains, except CO161, had significantly larger mean lesion sizes than any Pam isolate ($p < 0.001$). CO161 showed similar lesion size and symptom progression to Pam strains. Similar to the fruit inoculation test, this test also separated Pss and Pam (except for CO161) from *P.* spp. strains, which caused no lesions.

### 3.1.3 | Pathogenicity on Cherry Shoots

The smallest lesions (mean size of 1.0 mm, Figure 4) were observed on shoots inoculated with the negative control. Among the five *P.* spp. strains tested, the mean lesion size was 2.1–2.4 mm and there were no significant differences between the strains ($p = 0.778$). The mean size was 3.87–14.0 mm for lesions elicited by Pam and 5.65–13.68 mm for lesions elicited by Pss strains. Significant differences were observed within the Pss and Pam groups. Within the Pss group, strain CO231 had a significantly ($p < 0.001$) larger mean lesion size, of 12.4 mm, than strain CO23, at 6.1 mm. Similarly, Pam strain CO81 had a significantly ($p < 0.001$) smaller mean lesion size, of 4.8 mm, than strain CO44, at 14.0 mm. Differences were also observed between groups. For example, Pss strain CO231 had a significantly larger mean lesion size than Pam CO173 (12.3 vs. 5.9 mm; $p > 0.001$). The reverse was the case for strains Pss CO55 and Pam CO44 (mean lesions size 6.9 vs. 14.0 mm; $p < 0.001$). There was no significant difference in mean lesion size between Pss strains CO34 (9.3 mm) and CO150 (10.3 mm), or between Pam strains CO114 (9.9 mm) and CO179 (9.4 mm). Overall this test was able to separate non-pathogenic strains of *P.* spp. from Pss and Pam strains. However, variability among strains of Pss and Pam in their aggressiveness made separating the two pathovars not possible with this test.

### 3.1.4 | *Pseudomonas* Population Study on Detached Cherry Leaves

We inoculated nine representative *Pseudomonas* strains onto surface-sterilised cherry leaves and observed their growth over a 6-day period (Figure 5). The negative control did not yield any bacterial colonies. Among Pss strains, CO161, CO180 and ICMP21874 showed the greatest growth, with leaf macerates reaching $10^7$–$10^9$ cfu/mL at 6 dpi. In contrast, Pss strain CO23 showed significantly ($p < 0.001$) less growth, reaching $10^7$ cfu/mL at 6 dpi from a

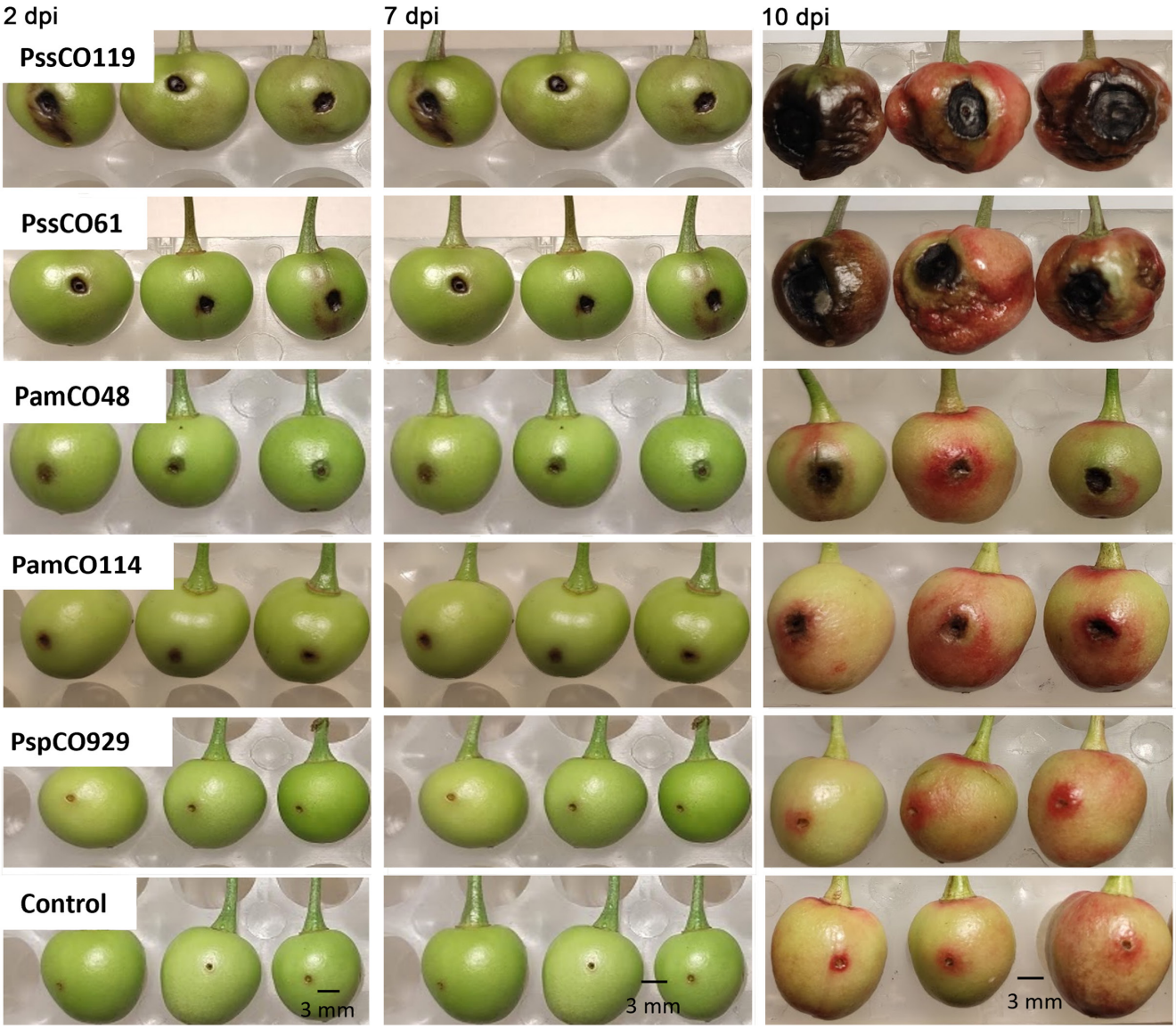


**FIGURE 2** | Symptom development on representative detached immature 'Lapins' cherry fruit following wound inoculation with a bacterial suspension ($1 \times 10^6$ cells/mL) of selected *Pseudomonas* strains (Marroni 2021) and incubated at 22°C. *Pseudomonas syringae* pv. *syringae* (Pss) PssCO119 and PssCO61; [*P. amygdali*. pv. *morsprunorum*] (Pam), PamCO114 and PamCO48; *P.* sp. CO929 and a negative control (10 mM $MgSO_4$), at 2, 7 and 10 days post inoculation (dpi). CO: Central Otago.

baseline of $10^4$ cfu/mL. Data from 6 dpi showed that the bacterial concentration in macerates of leaves infiltrated with ICMP21874 ($1.7 \times 10^9$ cfu/mL) was almost 100 times greater than for CO23 ($1.7 \times 10^7$ cfu/mL; $p < 0.001$) and more than three times greater than for CO161 ($4.4 \times 10^8$ cfu/mL; $p < 0.001$) and CO180 ($5.2 \times 10^8$ cfu/mL; $p < 0.001$). Meanwhile, *P.* spp. strains had significantly lower ($p < 0.001$ for all) growth rates than Pss strains, with leaf macerates reaching only $10^4$–$10^5$ cfu/mL at 6 dpi.

Pam strains CO48 and SVBPs6 increased from $10^4$ cfu/mL at baseline to $10^8$ cfu/mL at 6 dpi (Figure 5). At 3 dpi, the bacterial concentrations were similar among macerates of leaves infiltrated with the three Pam strains ($p > 0.99$). However, at 6 dpi, significant differences ($p = 0.008$) were apparent among Pam strains, with macerates of leaves infiltrated with SVBPs6 reaching $3.9 \times 10^8$ cfu/mL, a more than six-fold increase over CO114 ($6.5 \times 10^7$ cfu/mL) and ~1.5-fold increase over CO48 ($2.5 \times 10^8$cfu/mL). At both 3 and 6 dpi, macerates of leaves inoculated with *P.* spp. had significantly ($p < 0.001$ for both) lower cfu/mL ($10^4$–$10^5$ cfu/mL at 3 and 6 dpi) than leaves inoculated with Pam strains.

### 3.1.5 | Vascular Movement of Strains Inoculated Into Petioles of 'Lapins' Potted Plants

Inoculation of cherry leaf petioles with seven representative *Pseudomonas* strains resulted in vascular movement of bacteria to surrounding tissues. No bacterial colonies were recovered from the tissues of plants inoculated with the negative control. *Pseudomonas*-like colonies were consistently recovered in all three replicates across the various tissue segments taken from plants inoculated with Pss strains CO161, CO180 and ICMP21874 (Figure 6). Assessment at 14 dpi showed that the inoculation point (D) harboured the highest bacterial

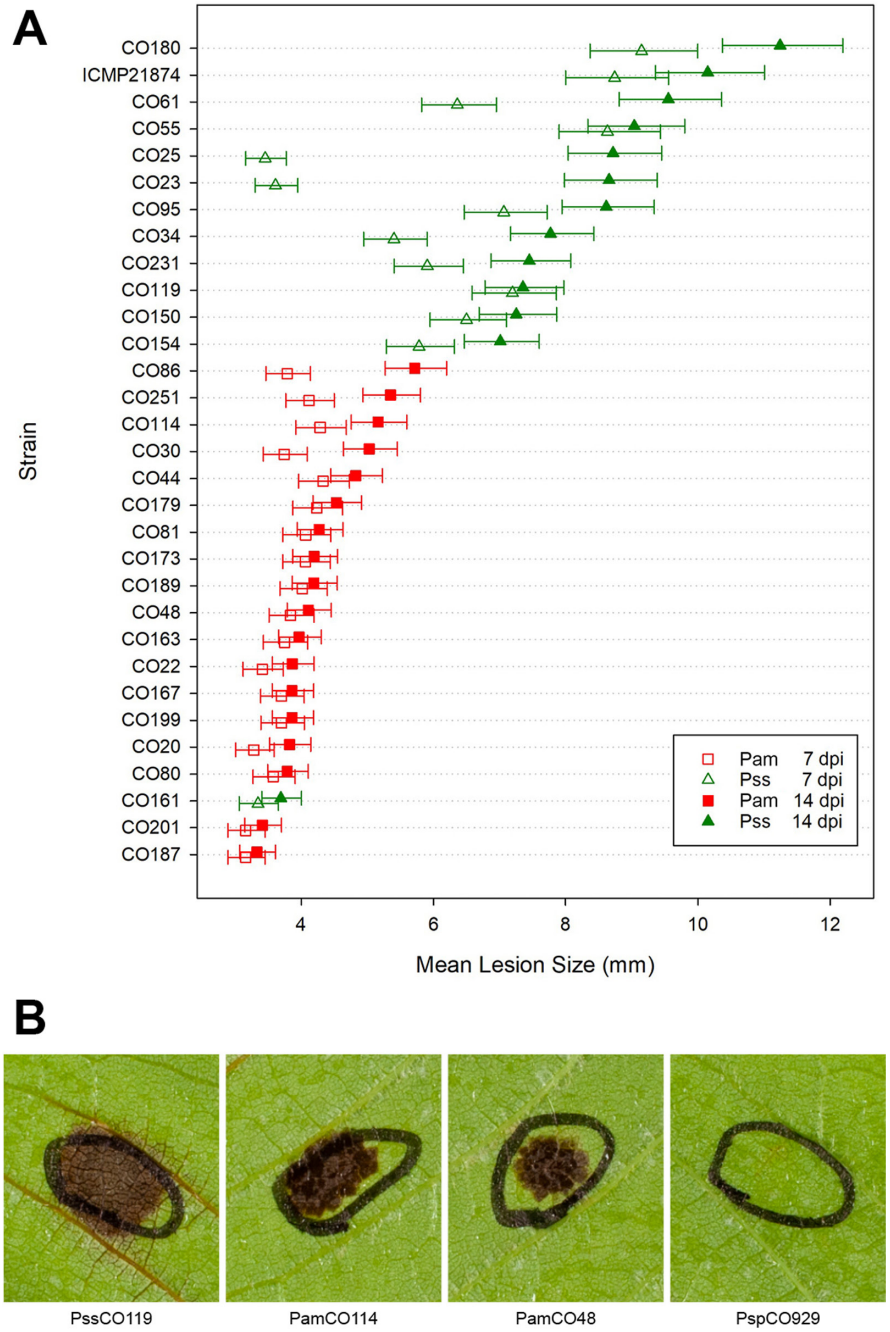


**FIGURE 3** | Pathogenicity on cherry leaves (Marroni 2021). (A) Mean lesion size developed on detached 'Lapins' cherry leaves following inoculation with *Pseudomonas* strains at 7 days post inoculation (dpi) and 14 dpi. New, fully expanded, detached cherry leaves were infiltrated by injecting 50–80 μL of a bacterial suspension ($10 \times 10^6$ cells/mL) of one of a subset of 35 strains of *P.* spp., *P. syringae* pv. *syringae* (Pss), [*P. amygdali* pv. *morsprunorum*] (Pam) and a negative control (10 mM $MgSO_4$), and then incubated at 22°C. Note that the negative control and the *P.* spp. strains did not produce lesions and therefore means are not shown. Error bars represent 95% confidence limits. CO, Central Otago. (B) Close up of lesion development 7 dpi by representative strains. PssCO119 is a strain Pss, note the necrosis that extended from the point of inoculation into the surrounding tissue and leaf veins; PamCO114 and PamCO48 are strains of Pam, note the necrosis restricted to the point of inoculation; PspCO929, is a strain of *P.* sp. (Psp), note that no symptoms developed.

concentration, which reached a mean of $10^7$ cells/mL for leaves treated with strains CO180 and ICMP 21874 and $10^9$ cfu/mL for leaves treated with CO161. Lamina segments showed a mean concentration of $10^4$ cfu/mL for all three strains. For petioles treated with CO161 and CO180, the mean bacterial concentration was $10^3$–$10^4$ cfu/mL in segments 1.0 and 2.0 cm above the inoculation point (segments E and F, respectively) and 1.0 and 2.0 cm below the inoculation point (segments G and H, respectively). For petioles inoculated with ICMP 21874, lamina segments also produced a mean $10^4$ cfu/mL, but this ranged from $10^7$ cfu/mL at 1.0 cm below the inoculation point (segment E) to $10^4$ cfu/mL at 2.0 cm above the inoculation point (segment

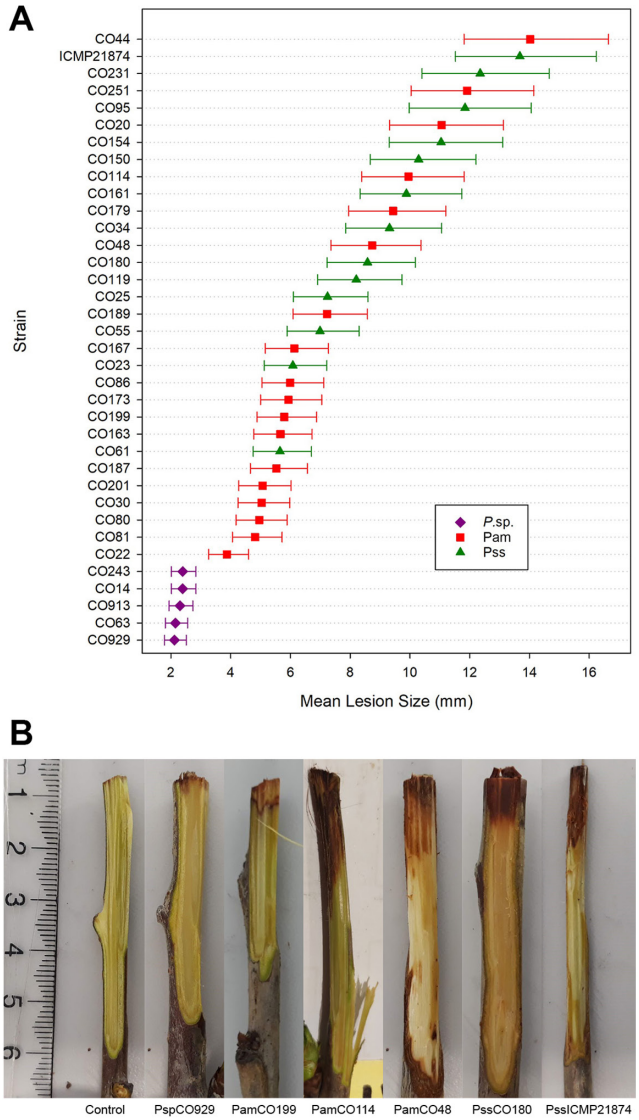


**FIGURE 4** | Pathogenicity on cherry shoots (Marroni 2021). (A) Mean length of lesions developed on detached one-year-old 'Lapins' cherry shoots after inoculation of the cut tips with a bacterial suspension ($10^8$ cfu/mL) of one of a subset of 35 *Pseudomonas* strains. The shoots were incubated at 15°C for 1 week, followed by −2°C for 1 week and then at 15°C for 4 weeks. Selected strains included: *Pseudomonas* spp., *P. syringae* pv. *syringae* (Pss), [*P. amygdali.* pv. *morsprunorum*] (Pam) and a negative control (10 mM $MgSO_4$). Error bars represent 95% confidence limits. CO, Central Otago. (B) Lesions developed by representative strains on one-year-old cherry shoots following inoculation with *Pseudomonas* strains. From left to right, Control; PspCP929 is a strain of *P.* sp.; PamCO199, PamCO114 and PamCO48 are strains of Pam; PssCO180 and PssICMP21874 are strains of Pss.

F) and 1.0 and 2.0 cm below the inoculation point (segments G and H). *Pseudomonas*-like colonies were recovered from all tissue segments examined after leaf inoculation with Pam strains CO48 and CO114 (Figure 6). At 14 dpi, the inoculation point (D) harboured the highest bacterial concentration (mean of $10^7$ and $10^8$ cfu/mL for CO114 and CO048, respectively), and the mean bacterial concentration in the lamina segments was $10^4$ cfu/mL. The mean bacterial concentrations above (segments

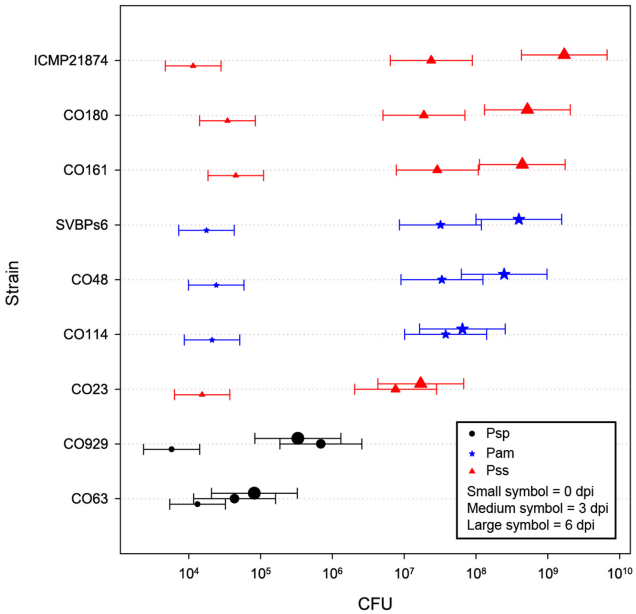


**FIGURE 5** | *Pseudomonas* population study on detached cherry leaves. Colony-forming units per mL (cfu/mL) recovered from leaf macerates obtained from detached, new fully expanded 'Lapins' cherry leaves infiltrated with a $10^5$ cfu/mL bacterial suspension of each of nine selected *Pseudomonas* strains: *Pseudomonas* spp., CO63 and CO929; *P. syringae* pv. *syringae* (Pss) CO23, CO161, CO180 and ICMP21874; [*P. amygdali* pv. *morsprunorum*] (Pam) CO114, CO48 and SVBPs6 and a negative control (10 mM $MgSO_4$). Measurements were taken at 0 days post-inoculation (dpi), 3 dpi, and 6 dpi and incubation at 22°C (Marroni 2021). Note that no colonies were recovered from macerates obtained from the Control and therefore results are not shown. Error bars represent 95% confidence limits. CO, Central Otago.

E and F) and below (segments G and H) the inoculation point were between $10^3$ and $10^4$ cfu/mL for both strains. Following petiole inoculation with *P.* spp. strains CO63 and CO929, *Pseudomonas*-like colonies were consistently recovered from the petiole and the three lamina segments. Bacterial concentrations reached $10^8$ cfu/mL in the petiole, $10^3$ cfu/mL at the tip of the lamina (segment A) and $10^4$ cfu/mL at the mid and base lamina (segments B and C) (Figure 6). Following petiole inoculation with CO929, there was no detectable bacterial migration to sections of the shoot 1.0 or 2.0 cm below (segments G and H, respectively) or above (segments E and F, respectively) the inoculation point (D). In contrast, *Pseudomonas*-like colonies ($10^2$ cfu/mL) were recovered 1.0 cm above the inoculation point (segment E) after petiole inoculation with CO63.

## 3.2 | Virulence-Associated Factors

### 3.2.1 | Hypersensitive Reaction on Tobacco Leaves

We tested 35 strains for their pathogenicity on tobacco plants. Pss and Pam strains tested positive, indicating their ability to cause disease. In contrast, *P.* spp. strains did not elicit a hypersensitive response in tobacco, showing they were not potential phytopathogenic bacteria.

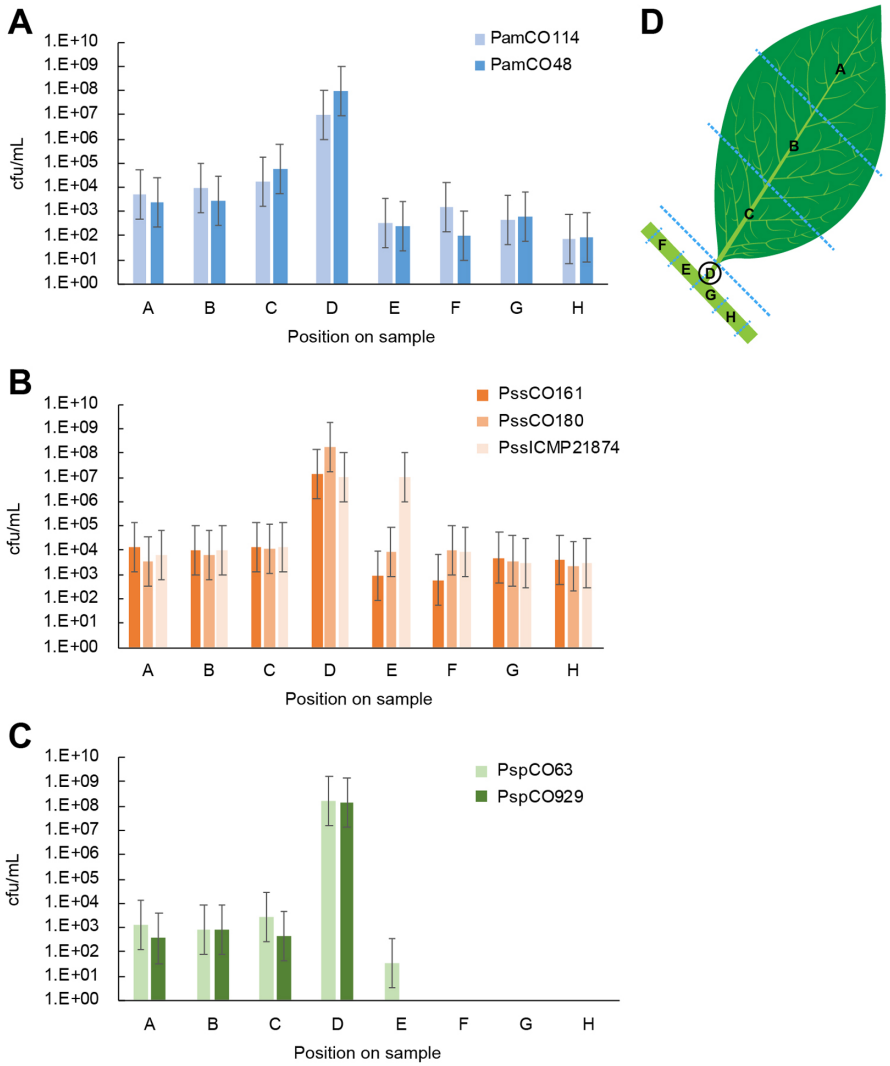


**FIGURE 6** | Bacterial colony-forming units (cfu)/mL obtained from sections of the lamina, petiole and shoot after petiole-inoculation of leaves of 'Lapins' potted cherry plants with selected *Pseudomonas* strains (Marroni 2021). The petioles of new, fully expanded leaves were puncture-inoculated with a bacterial suspension of $10^6$ cfu of each of (A) [*P. amygdali* pv. *morsprunorum*] (Pam) CO114, CO48; (B) *P. s.* pv. *syringae* (Pss) CO161, CO180 and ICMP21874 and (C) *P.* spp. (Psp) CO63 and CO929. A negative control (10 mM $MgSO_4$) was also included. (D) Diagram showing inoculation and sampling sections. After 14 days incubation at 22°C, subsamples were taken from the lamina (sections A, B and C for apex, mid and base sections, respectively), the petiole (section D, inoculation point) and from 1 and 2 cm sections above the petiole (sections E and F, respectively) and below the petiole (sections G and H).

### 3.2.2 | Production of Phytotoxins: Syringomycin and Coronatine

Table 1 summarises the phytotoxin results for the 35 selected strains, while Table S1 includes results for all tested strains. Of the 250 strains tested for coronatine-encoding genes, only one Pam strain (1/70, 1.4%) tested positive (CO114). Of the 153 Pss strains, 88% (134/153) tested positive for syringomycin production. Two of 27 *P.* spp. strains produced syringomycin, while no Pam strains produced this toxin.

### 3.2.3 | Ice Nucleation Activity

Of the strains classified by Marroni et al. (2023) as Pss, 97% (147/153) were Ice+. Seventy Pam strains and 27 *P.* spp. strains were included in the INA analysis, none of which were found to catalyse ice nucleation at −5°C. Table 1 summarises the INA findings for the selected subset of 35 strains and Table S1 presents INA findings for the full set of 250 strains.

## 4 | Discussion

This study found significant differences in aggressiveness and virulence-associated factors among strains of Pss, Pam and *P.* spp. from Central Otago cherry orchards. The results supported the previous classification of strains (Marroni et al. 2023) into two major taxonomic groups (Pss in PG2, Pam in PG3 and non-pathogenic strains).

Both Pss and Pam coexist in orchards in Central Otago, but Pss is the prevalent strain across tissue types and locations (Marroni et al. 2023). Although the reasons for the prevalence of Pss are

not entirely clear, gaining insight into the distinct characteristics of both Pss and Pam strains, as well as their interactions with host plants, can offer valuable understanding regarding the factors influencing their relative abundance. In this study, pathogenicity on cherry was demonstrated for all Pss and Pam strains, but their aggressiveness varied depending on the tissue studied. Except for CO161, strains of Pss caused faster appearance and more severe symptoms on fruit and leaves than strains of Pam, similar to the results reported by Hulin et al. (2018) and Kaluzna and Sobiczewski (2010) on fruit. The observation that CO161 lacked syringomycin production and ice nucleation activity (INA) suggests that these virulence factors may play a role in the reduced pathogenicity of this strain. More detailed characterisation of CO161's whole genome could enhance accurate identification and exclude the presence of previously non-reported strains in New Zealand. Strains of Pss and Pam demonstrated an ability to move systemically within the plant, as evidenced by their ability to increase and maintain their populations on detached cherry leaves. In addition, the recovery of *Pseudomonas*-like colonies from the inoculated petiole study suggested that Pss and Pam strains were able to move systemically within the plant, as previously reported in *Prunus* species (Roos and Hattingh 1987; Hulin et al. 2018) and kiwifruit for *P. s* pv. *actinidiae* (Psa) (Ferrante and Scortichini 2014). In addition, for a limited number of non-pathogenic strains, their persistence, growth and movement within cherry tissue was also demonstrated (albeit to a lesser extent than observed in Pss and Pam strains).

Differences in disease expression may reflect variability in the set of virulence factors of strains, which in turn can affect niche colonisation, persistence and severity of symptoms (Mo and Gross 1991; Scholz-Schroeder et al. 2001; Hulin et al. 2018; Ruinelli et al. 2019) and may help to explain the prevalence of Pss strains in Central Otago orchards. In this study, for both Pss and Pam strains, a functional T3SS was inferred, given the positive hypersensitivity reaction on tobacco leaves (Xin, Kvitko, and He 2018) and the symptoms on cherry tissue. A more comprehensive investigation into the T3SS of Pss and Pam strains from Central Otago orchards is essential. This research will not only deepen our understanding of the molecular mechanisms driving pathogenicity but also offer valuable insights into the specific virulence strategies utilised by these strains in cherry tissue. Two other virulence factors, the production of phytotoxins and INA, were also explored. Among the Pss strains, syringomycin production was prevalent (88%). This phytotoxin is associated with membrane disruption, ion leakage and severe symptoms associated with a more necrotrophic lifestyle (Scholz-Schroeder et al. 2001; Hulin et al. 2020). In addition, it contributes to the competitive fitness of syringomycin-producing strains in the apoplast and inhibition of growth of other microorganisms (Lavermicocca et al. 1997; Helmann, Deutschbauer, and Lindow 2019). In contrast, the vast majority of Pam strains in this study did not produce coronatine. Coronatine is a non-host specific phytotoxin, produced by some Pam strains and related to the suppression of host defences and chlorosis of plant tissue (Brooks, Bender, and Kunkel 2005; Xin, Kvitko, and He 2018). Strains of Pam that do not produce coronatine have been reported before (Mitchell 1982), with the possession of coronatine genes considered as a discriminating factor that separates Pam strains with high and low aggressiveness on cherry (Gilbert et al. 2009; Hulin et al. 2018). In this single-strain inoculation study, Pam strains achieved similar population growth on inoculated leaves as Pss and elicited necrotic lesions on fruit and shoots. These findings demonstrate that the absence of coronatine was not a limiting factor for host colonisation by Pam. Pam is known to use a larger set of effectors and is less reliant on the production of toxins to infect and colonise its host than Pss (Xin, Kvitko, and He 2018). In contrast, Pss is reported to possess a small set of effectors that is compensated for by the production of phytotoxins (Baltrus et al. 2011; Xin, Kvitko, and He 2018). Nevertheless, the lack of coronatine may render Pam strains less competitive in plant tissue (Bender et al. 1987) in the presence of Pss syringomycin-producing strains, which may occur in the natural environment.

Most Pss strains tested in this study were able to catalyse ice formation. Frost damage is a well-known predisposing factor for the development of bacterial canker in *Prunus* (Lindow 1983; Sobiczewski 1992; Kennelly et al. 2007), providing an entry point for *Pseudomonas* strains. An association between frost damage and increased disease incidence is not exclusive to Ice+ *Pseudomonas* strains. When Pss, Pam or both are present, the incidence and severity of bacterial canker on *Prunus* species is higher in regions with a continental climate, with the occurrence of spring and winter frosts and lower in Mediterranean areas (Kennelly et al. 2007). Frost events and Psa, an Ice- strain, have been associated with a higher incidence of bacterial canker on kiwifruit in Italy, New Zealand and China (Ferrante and Scortichini 2014; Froud et al. 2015). Frost alone can increase disease severity by inducing tissue wounds that provide an entry point for colonisation by *Pseudomonas* strains. However, Ice+ Pss strains may play a more active role and contribute to frost damage by limiting the ability of the host tissue to supercool and avoid ice formation at temperatures just below 0°C, resulting in tissue damage (Lindow 1983; Montesinos and Vilardell 1991). While the frequent frosts in Central Otago orchards through autumn and spring provide abundant opportunities for damaged plant tissues to be colonised by Pss and Pam, the prevalence of Ice+ Pss strains may exacerbate disease development.

Nonpathogenic *P.* spp. and pathogenic Pss and Pam strains coexist in the same orchard in Central Otago (Marroni et al. 2023). The co-existence of pathogenic and/or nonpathogenic strains, whether from the same or different genera, within the same tissue type, suggests the possibility of interactions between these organisms that could potentially influence disease expression (Lamichhane and Venturi 2015; Bophela et al. 2020). Indeed, several studies have shown that the presence of non-pathogenic strains can affect the virulence and competitiveness of pathogenic strains, by either stimulating or inhibiting their growth (Blakeman and Fokkema 1982; Wilson and Lindow 1993; Pusey, Stockwell, and Mazzola 2009). Moreover, non-pathogenic strains may act as reservoirs of genetic diversity, which could eventually lead to the emergence of new virulent strains through recombination or gene exchange (Monteil et al. 2013; Hulin et al. 2020, 2023). For instance, horizontal gene transfer has been shown to contribute to the acquisition of virulence factors in several bacterial pathogens, including *Pseudomonas syringae* (Baltrus et al. 2011). Therefore, the co-existence of pathogenic and

non-pathogenic strains within the same host tissue may represent an opportunity for genetic exchange and the emergence of new pathogenic variants (Hulin et al. 2023).

In summary, this study provides valuable insights into the pathogenicity and dynamics of Pss, Pam and *P.* spp. strains in Central Otago cherry orchards. The observed level of genetic and phenotypic diversity among Pss, Pam and *P.* spp. strains highlights the complexity of microbial populations within Central Otago cherry orchards. This diversity manifested in a spectrum of pathogenicity, ranging from highly virulent to less virulent strains like CO161 to non-pathogenic strains. The coexistence of strains with varying degrees of virulence and non-pathogenic strains suggests a complex ecological balance, where multiple factors, including genetic variation, virulence traits and environmental conditions, shape the population dynamics and disease outcomes.

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**Conflicts of Interest**

The authors declare no conflicts of interest.

**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Peer Review**

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/jph.13390.

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section.