

Absence of *Pseudomonas syringae* pv. *actinidiae* in kiwifruit leaves and flowers from Buenos Aires Province, Argentina.

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Abstract

Argentina has around 1000 ha of kiwifruit orchards in Buenos Aires Province. This country is recognized as free of *Pseudomonas syringae* pv. *actinidiae* (Psa). In 2013, the National Sanitary Service (SENASA) imposed measures to avoid the entrance of this pathogen to the kiwifruit production areas. In this way, a vigilance plan was established and the introduction of plant material was regulated. In March 2015, three infected samples with Psa were detected in orchards at the South-East of Buenos Aires Province and they were immediately eradicated by SENASA. The objective of this work was to identify the bacteria associated with kiwifruit leaf spots and flower blights in commercial orchards of *Actinidia deliciosa* 'Hayward' at Buenos Aires Province, during the 2015-2016 growing season. On spring and autumn seasons, plant tissues were collected from a 100 ha area located in the North and South-East of this Province. Bacterial isolates were obtained from flowers and leaves by tissues mashing and inoculation on nutrient agar supplemented with 5% sucrose (NSA). The primary identification of isolates was performed by biochemical and physiological tests. To confirm their identity, duplex-PCR tests were performed, including negative and positive controls (no template and Psa DNA, respectively). From symptomatic and asymptomatic samples, 120 bacterial isolates were obtained. Thirty of these isolates were morphologically similar to Psa, forming white-creamy, convex, domed and mucoid colonies on NSA. Also, all of them were Gram-negative, produced a green fluorescent pigment on King's B medium and their metabolism was aerobic. However, none of these strains examined produced any bands following the amplification by PCR with Psa specific primers. Our results show the absence of Psa in the analyzed samples. Further studies are needed to identify these non-Psa bacteria associated with kiwifruit leaf spots and flower blights.

Keywords: *Actinidia deliciosa*, 'Hayward', bacterial canker, quarantine pest

INTRODUCTION

The kiwifruit (*Actinidia deliciosa* (A. Chev.) C. S. Liang et. A.R. Ferguson) production has had a sustainable growth during last decade in Argentina. The main area of this crop is concentrated in the South-East of Buenos Aires Province (departments of Balcarce, General Alvarado, General Madariaga, General Pueyrredón and Mar Chiquita) and at the North (departments of Baradero, La Plata, Mercedes and San Pedro) (Benés *et al.*, 2014). At the South-East of Buenos Aires Province, there is an area of 440 hectares of kiwifruits orchards, representing around 50% of the national production (Yommi, *pers. commun.*). Most of the national area is planted with the kiwifruit 'Hayward' that has excellent postharvest attributes and organoleptic characteristics (Rushing, 2004).

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Pseudomonas syringae pv. *syringae* (Pss), *P. syringae* pv. *actinidiae* (Psa), *P. viridiflava* and *P. marginalis* can cause necrotic and angular leaf spots, blossom blights and twigs cankers on kiwifruit (Wilkie *et al.*, 1973; Young *et al.*, 1988; Takikawa *et al.*, 1989; Balestra and Varvaro, 1997; Mansilla and Abelleira, 1999; Gonzales and Rodicio, 2007; Balestra *et al.*, 2010; Gallelli *et al.*, 2011b). Psa kiwifruit bacterial canker is the most destructive and severe kiwifruit's disease (Donati *et al.*, 2014). This bacterium was reported in most of the kiwifruit areas of the world (Scortichini *et al.*, 2014). Severe epidemics and yield losses were reported in Chile, Italy, Japan, Korea, New Zealand, Portugal and Spain (Donati *et al.*, 2014).

Actinidia spp. are the only known host plant species of Psa. These bacteria can be detected on both symptomatic and symptomless plants (Mazzaglia *et al.*, 2010), in latently infected aerial plant parts and contaminated pollen (Vanneste *et al.*, 2011). Infected plants and pollen are considered an essential way of the spread of the disease. The rain-splash and wind were the main mechanisms of the natural spread between and within vines (Tyson *et al.*, 2014). The symptoms are present in aerial parts such as trunks, leaders, canes, leaves and flowers (Scortichini *et al.*, 2012). The most typical symptoms of Psa are shoot-wilt; defoliation and dieback, with the presence of white to reddish-brown exudates through the cankers. The leaves show small, angular, water-soaked areas which can later become necrotic and turn dark brown; a chlorotic halo is commonly observed around each necrotic spot. Similar symptoms might be caused on leaves by Pss. Also *P. viridiflava* has been reported as causing leaf necrotic spots, but without chlorotic halos (Scortichini, 1994; Balestra *et al.* 2010; Abelleira *et al.*, 2011; Everett *et al.*, 2011; Ferrante y Scortichini, 2009; Gallelli *et al.*, 2011b). Psa causes browning of the sepals, flowers become necrotic and then can fall. In severe attacks, fruits can be dehydrated and reduce its development. All these symptoms cause important yield losses (KVH, 2011).

The genotypic, biochemical, and pathogenic variability of Psa was studied. Biovar 1 has been associated with severe epidemic events in Japan and Italy. Biovar 2 was reported only affecting orchards in South Korea. Pandemic strains of biovar 3 caused severe losses in Chile, Italy, New Zealand and China. Strains of these three biovars are considered virulent causing necrotic leaf spotting, canker and shoot die-back (Chapman *et al.*, 2012; Vanneste, 2013; Ferrante and Scortichini, 2015). Biovar 4, described in Australia and New Zealand, is less virulent and was recently renamed as *Pseudomonas syringae* pv. *actinidifoliorum* pv. *nov.* (Cunty *et al.*, 2015). These authors reported that only necrotic spots on leaves are the symptoms associated with this new pathovar.

In Argentina Psa is a quarantine pest, classified as A1. At 2013, the National Sanitary Service (SENASA, Servicio Nacional de Sanidad y Calidad Agroalimentaria) established a phytosanitary alert through the 589/2013 Resolution. In this way, the entrance of any kiwifruit vegetal parts was prohibited to avoid the introduction and dispersion of the pathogen. Since this moment, SENASA monitors orchards and nurseries with a systematic scheme of controls. In 2015, three positive samples at the South East of Buenos Aires Province were detected and those plants were immediately eradicated (SENASA, 2015).

The objective of this work was to identify the bacteria associated with kiwifruit leaf spots and flower blights in commercial orchards of *A. deliciosa* 'Hayward' at Buenos Aires Province, during the 2015-2016 growing seasons.

MATERIAL AND METHODS

Sampling

Across the 2015 spring and the 2016 autumn, plant tissues were collected from a 100 ha area located in the North and South-East of Buenos Aires Province. Every orchard was inspected with a systematic and complete path. The whole group of plants on both sides of a line was examined (one line every three lines), taking a sample from a plant every 10 plants in the left side of the line. Leaves, sprouts and flowers (healthy and symptomatic) were collected. The samples were conditioned to be carried to the

laboratory in plastic bags inside a cooled basket, and they were stored at 4°C for no more than 3 days before the analysis.

Isolation

The isolation was performed from macerated tissues in sterilized saline solution (0.85 % w:v, sodium chloride:distilled water). Ten-fold dilutions were performed and the three last dilutions were streaked on Sucrose Nutrient Agar (SNA) (Kado and Heskett, 1970). The plates were incubated two days at 24 °C. Macroscopic characteristics of colonies growing on SNA were assessed (length, form, arrangement, and Levan production). On King's B medium (King *et al.*, 1954) the production of fluorescent pigments was detected.

Primary identification

The biochemical and metabolic profiles of the isolates were determined by the protocols established by Standard EPPO Diagnostics PM 7/120(1) (2014).

PCR identification

The identification of those isolates with biochemical and physiological profiles similar than the species *Pseudomonas syringae* was confirmed through PCR. A duplex-PCR reaction for detection of DNA specific fragments of Psa was performed to differentiate between pathovars *syringae* and *actinidiae* (Gallelli *et al.*, 2011a).

RESULTS AND DISCUSSION

On October and November of 2015 six kiwifruits orchards were monitored, one in Balcarce, one in Gral. Alvarado and four in Gral. Pueyrredón. On April and May of 2016, tissue samples were collected in two orchards of La Plata, five orchards in Gral. Pueyrredón and another one in Balcarce (Fig. 1).

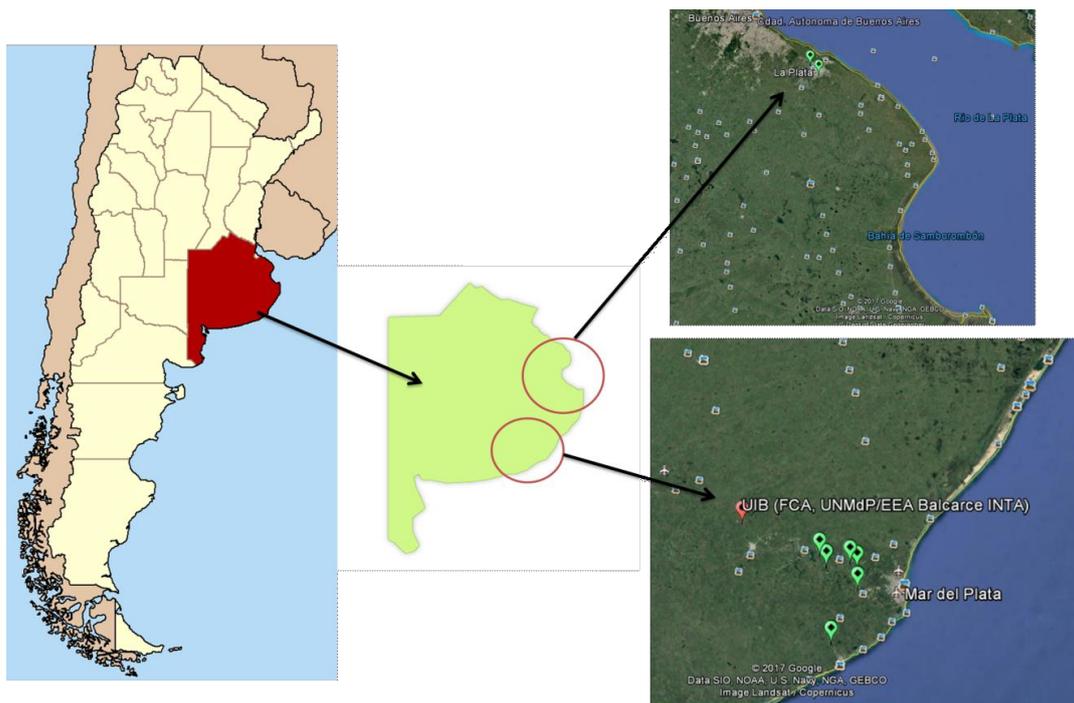


Figure 1. Location of the monitored orchards in the Buenos Aires Province, Argentina. The red mark indicates the location of our work place, where there is an experimental kiwifruit orchard too.

A total amount of already 200 tissue samples were collected in this study. One hundred and twenty isolates showing morphological characteristics described for *P. syringae*, were obtained from symptomatic organs and tissues without symptoms. The observed symptoms were small necrotic spots, angular in shape, some with chlorotic

halos, necrotic and aqueous spots in sepals, as shown in Figure 2. The origin and the distribution of the isolates, according to the time of sampling and the organ where were isolated are shown in Table 1.

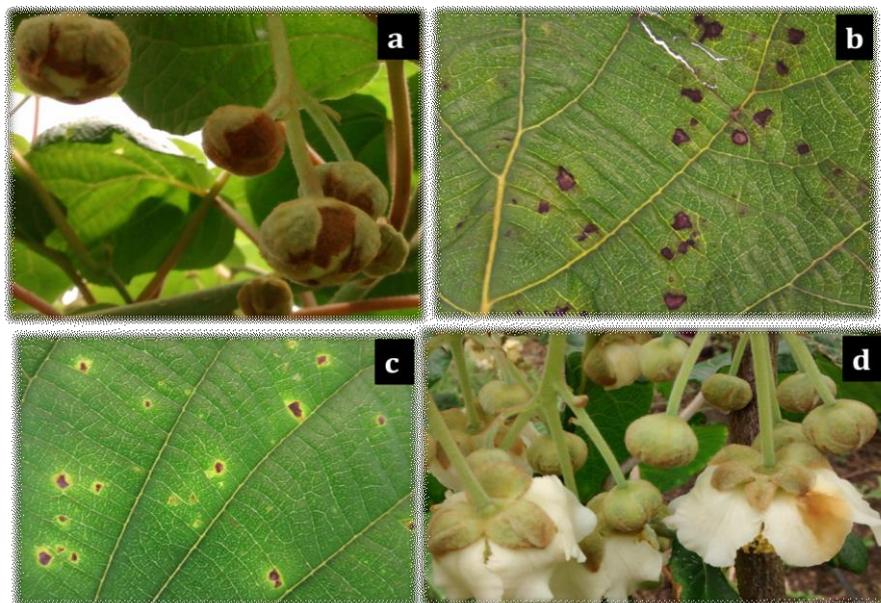


Figure 2. Different symptoms observed on the monitoring orchards of *Actinidia deliciosa* 'Hayward' at Buenos Aires Province, during 2015-2016 growing seasons. (a) necrotic spots in sepals; (b) small angular leaf spots without chlorotic halos; (c) angular leaf spots surrounded by chlorotic halos and (d) necrotic petals on flowers.

Table 1. Distribution of the isolates, according to the time of sampling and the part of the plant where were isolated.

Monitoring season	Location	Vegetable organ	Isolets
2015 Autumn	SE Bs As	Leaves	15
	Gral Alvarado	Leaves	1
		Flower buds	2
	Batán, Gral Pueyrredón	Leaves	2
		Flower buds	4
2015 Spring	El Dorado, Gral Pueyrredón	Leaves	5
		Flower buds	1
	Sierra de los Padres, Gral Pueyrredón	Flowers	3
		Leaves	6
	Balcarce	Leaves	0
		TOTAL 2015	39
2016 Autumn	La Plata	Leaves	29
	Batán, Gral Pueyrredón	Leaves	23
	El Dorado, Gral Pueyrredón	Leaves	13
	Sierra de los Padres, Gral Pueyrredón	Leaves	11
	Balcarce	Leaves	5
		TOTAL 2016	81
		TOTAL	120

Seventy out of 120 isolates were Gram's negative and showed an oxidative metabolism of glucose. These isolates were considered presumptive *Pseudomonas* spp. (Goszczyńska *et al.*, 2000). Those isolates which showed Levan production in SNA medium (fluorescents and non-fluorescents) were classified as possible isolates of *P. syringae* (Takikawa *et al.*, 1989; Scortichini, 1994). Thirty out of the 70 isolates showed the characteristics reported by Ferrante and Scortichini (2009), Everett *et al.* (2011) and EPPO (2014) for *P. syringae*. These colonies were greyish, pearly coloured, convex, circular with defined borders (Fig. 3). The isolates that were fluorescent but did not produce Levan were considered as *P. viridiflava* (Wilkie *et al.*, 1973; Young *et al.*, 1988; Balestra and Varvaro, 1997). *P. marginalis* can be differentiated from the other *Pseudomonas* species by positive reactions of oxidase and negative reaction in the hypersensitive tobacco test (Mansilla and Abelleira, 1999; Gonzales and Rodicio, 2007).

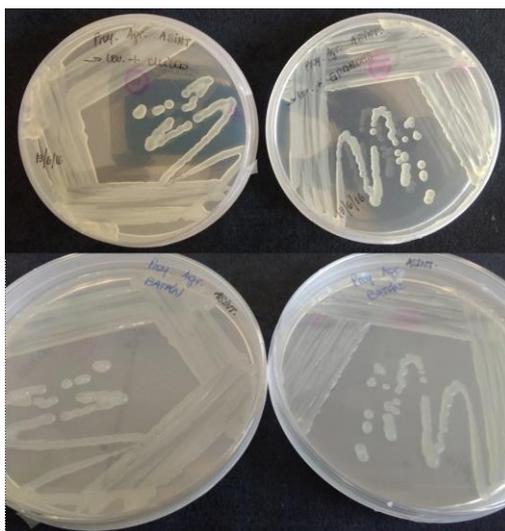


Figure 3. Some of the thirty colonies obtained from symptomatic leaf tissues, classified as possible isolates of *P. syringae*. There were greyish, pearly coloured, convex and circular with defined border colonies.

The *Pseudomonas* fluorescent group can be classified through LOPAT probes: Levan production, oxidase, potato soft rot, arginine dihydrolase and HR on tobacco (Goszczyńska *et al.*, 2000). These tests are useful for a primary characterization of the isolates, but they are not sufficient to differentiate between Psa and Pss. Ferrante and Scortichini (2009) and Everett *et al.* (2011) described the morphology of Psa colonies in SNA medium as pearly to white, smooth, convex, circular, with defined borders.

Thirty isolates presumptive considered as *P. syringae* pathovars were analysed by duplex-PCR reaction to detect if they belong to Psa or Pss (Gallelli *et al.*, 2011a). None of them showed the predicted PCR products of 492 bp and 230 bp, respectively (Fig. 4). These results indicate the absence of Psa in the group of analysed samples.

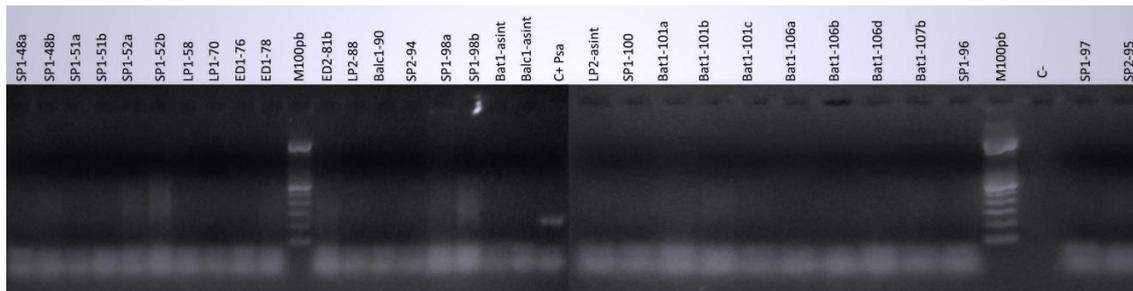


Figure 4. Gel electrophoresis analysis of duplex-PCR amplification by using primers KN-F/R and Avr/DdxF/R (Gallelli *et al.*, 2011a). Psa positive and negative controls were included (C+ Psa and C-, respectively).

Psa is the causal agent of the most damaging and severe disease of cultivated kiwifruits, but their symptoms are very similar to other *Pseudomonas* species reported on kiwifruit orchards. Further identification of these bacteria must be achieved.

CONCLUSIONS

- *Pseudomonas* species were isolated from kiwifruit leaf spots and flower blights in orchards of *A. deliciosa* 'Hayward' at Buenos Aires Province, demonstrating the presence of these pathogens in the studied area.
- Psa is not present in the analysed samples of kiwifruit from commercial orchards during the 2015-2016 growing seasons.
- Further studies are needed to identify the *Pseudomonas* species isolates.

ACKNOWLEDGMENTS

This work has been financially supported by Univ. Nacional de Mar del Plata, Argentina (UNMDP) (projects AGR517/16 UNMDP), Asoc. Coop. de la Fac. de Cs. Agrarias (FCA, UNMDP) and the Instituto Nac. de Tecnología Agropecuaria (INTA) (projects PNFRU1105083 and BASUR1272103). We wish to thank to Comisión de Investigaciones Científicas de la Prov. de Buenos Aires (CIC). The present work constitutes part of the Doctoral thesis of the first author, María Clara Sanchez, at the FCA-UNMDP.

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