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Bacteria from four phylogroups of the *Pseudomonas syringae* complex can cause bacterial canker of apricot.

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Running head: Etiology of bacterial canker of apricot

Keywords. Stone fruits, *Prunus armeniaca*, bacterial canker disease, *Pseudomonas syringae* species complex.

Abstract

Pseudomonas syringae is described as a species complex, containing *P. syringae*-related species classified into 13 phylogroups and 23 clades. *P. syringae* is one of the main pathogens of fruit trees, affecting nut trees, hazelnut and kiwi, pome and stone fruits. Bacterial canker of apricots is an important disease in regions of production with cold winters and conducive soils. In this work, we characterized the bacteria able to induce canker in apricots isolated in different French orchards. Bacteria from four phylogroups were able to induce canker. The pathogenicity to apricot was not linked to the pathogenicity to

the three herbaceous species and cherry fruits tested, and was not always related to This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ppa.13051

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Parisi, L. (Auteur de correspondance), Morgaint, B., Blanco Garcia, J., Guilbaud, C., Chandeysson, C., Bourgeay, J.-F., Moronvalle, A., Brun, L., Brachet, M. L., Morris, C. E. (2019). Bacteria from four phylogroups of the Pseudomonas syringae complex can cause bacterial canker of apricot. Plant Pathology. 68 (7), 1249–1258. DOI : 10.1111/ppa.13051 hypersensitive reaction on tobacco and ice nucleation activity. Bacteria pathogenic to apricot belong to phylogroups 01, 02, 03 and 07. The bacteria of phylogroups 01a and 07a (*P. viridiflava*) characterized in this work were not described previously as pathogenic to apricot.

Introduction

Pseudomonas syringae is described as a species complex, composed of a phylogenetic lineage containing *P. syringae*-related species (Mulet *et al.* 2010) together with *P. syringae* populations classified into 13 phylogroups (PG) and 23 clades (Berge *et al.* 2014). *P. syringae* complex comprises strains involved in various crop diseases: more than 60 pathovars of *P. syringae* have been described, infecting almost all economically important crop species (Lamichhane *et al.* 2014, 2015). In addition, disease outbreaks, caused by new *P. syringae* strains, continue to threaten global crop production. As part of the complex ecology of this group, many *P. syringae* strains have also been frequently isolated all along the water cycle both in and outside of agricultural zones and from a range of substrates (Morris *et al.* 2008, Monteil *et al.* 2013).

Over the last 10 years, *P. syringae* has become a major pathogen of fruit trees, affecting nut trees, hazelnut and kiwi, in addition to pome fruits and *Prunus spp.* (cherry, peach, plum and apricot) that have been known as hosts of the pathogen for a long time (Lamichhane *et al.*, 2014). Bacterial canker of stone fruits is a vascular disease characterized by the systemic movement of the bacteria throughout the plant and in particular the vessels (phloem and xylem). It is often devastating, causing sudden wilting and dieback, presence of extended cankers on stems, branches and main trunks, production of exudates and gummosis

(Lamichhane *et al.*, 2014). The incidence of bacterial canker is reported to be 50-100% on wild cherry in the UK (Vicente *et al.*, 2004), 50% on plum in the Netherlands (Wenneker *et al.* 2012) and 80% on apricot in Turkey (Kotan & Sahin, 2002). In Europe in general, bacterial canker is one of the most important diseases of apricot in the regions with spring frost conducive for the disease.

For the implementation of effective control measures of a disease, it is necessary to have good knowledge of the pathogen, its life cycle and especially inoculum reservoirs. The originality of *P. syringae* as pathogen of stone fruit trees is that different strains, belonging to different PGs of the *P. syringae* complex, can be involved in the development of disease; this can make it more difficult to control disease, as the different pathogenic strains representing a great genetic variability can be adapted to different hosts, cultivars and pedoclimatic conditions. Moreover, as bacteria of the *P. syringae* complex are present in many different substrates (water, litter, residues, grasses and ground-covers) of cultivated and non-cultivated habitats, the question of the emergence of pathogenic strains from these meta-populations is crucial (Morris *et al.* 2009).

Currently, five different members of the *P. syringae* group belonging to four different phylogroups described by Berge *et al.* (2014) are known to be pathogenic to stone fruits (Bultreys & Kaluzna, 2010). In PG01, *P. syringae* pv. *avii* has been reported to be pathogenic to wild cherry, *P. syringae* pv. *morsprunorum* race 2 to cherry and *P. syringae* pv. *persicae* to peach, nectarine and Japanese plum. In PG02, *P. syringae* pv. *syringae* was described in cultivated and wild cherry, plum, peach and apricot. In PG03, *P. syringae* pv. *morsprunorum* race 1 was pathogenic to cherry, plum and apricot.

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P. syringae pv. *morsprunorum* race 1 and 2 were first described by Wormald (1931) and Freigoun & Crosse (1975), but naming these two bacteria race 1 and 2 is probably not appropriate. Currently, it is known that they belong to different PGs (PG01 and PG03) and it is clear that they are two distinct genetic lines. They are now named *P. amygdali* pv. *morsprunorum*, with pathotype strain MAFF302280PT in PG01 and reference strain FTRS U7805 in PG 03 (Gomila *et al.*, 2017).

In France, bacterial canker of apricots is an important disease in regions of production with cold winter and conducive soils that are generally light, sandy soils with fluctuating drought conditions (Young, 1987). Mid-mountain regions with alternating night frost and diurnal melting are highly impacted by the disease, due to the ice nucleation activity (INA) of several lines of the *P. syringae* group (Lindow, 1983). This is the case in Rhône-Alpes historical region, which represents a great part of the apricot production in France (58% of French production in 2017). Bacterial canker of apricot is also an important disease in regions of Kurdistan in Iran, in Turkey, in Bulgaria and in Italy (Karimi-Kurdistani & Harighi 2008, Kotan & Sahin 2002, Donmez *et al.* 2010, Inanova, 2009 and Scortichini, 2006).

The objective of this work was to identify the members of *P. syringae* complex causing the bacterial canker of apricot in France. For this purpose, we first estimated the frequencies of the different PGs of the *P. syringae* complex on a large sample of strains obtained from canker symptoms in orchards. Then we characterized a sub-set of strains for induction of hypersensitivity in tobacco, ice nucleation activity, as components of the bacterial pathogenicity. The production of syringomycin-like toxins was also evaluated for the PG02 strains, because the production of these necrosis-inducing toxins is described in bacteria of this PG (Gross, 1991). Pathogenicity to *Prunus armeniaca* for some of these strains was also

This article is protected by copyright All rights reserved. Parisi, L. (Auteur de correspondance), Morgaint, B., Blanco Garcia, J., Guilbaud, C., Chandeysson, C., Bourgeay, J.-F., Moronvalle, A., Brun, L., Brachet, M. L., Morris, C. E. (2019). Bacteria from four phylogroups of the Pseudomonas syringae complex can cause bacterial canker of apricot. Plant Pathology, 68 (7), 1249–1258. DOI: 10.1111/ppa.13051 evaluated, like the pathogenicity to a set of herbaceous hosts, to explore the possibility to predict the pathogenicity to apricot. This work was part of a research program devoted to the identification of reservoirs of *P. syringae* in apricot orchards, to improve knowledge on epidemiology of the disease.

Materials and methods

Isolation of bacterial strains

In this work, we first obtained a large number of strains (779) for molecular characterization with PCR, then we made a subset of 30 purified strains representing the full diversity of the initial set, for more precise phenotypic characterization.

The 779 strains of the *P. syringae* complex were all obtained from symptomatic trees of *P. armeniaca,* from two different origins: 530 strains originated from an INRA experimental station in Drôme County, near Valence, France, called the "Unité Expérimentale de Recherches Intégrées) (UERI) and 249 strains originated from a survey made by field technicians of the "Centre Technique Interprofessionnel des Fruits et Légumes" (CTIFL) in 2011-2012 in commercial orchards along the Rhône Valley, in the east-southern part of France.

P. armeniaca trees from eight experimental plots (15 trees) expressing canker symptoms were sampled on 13 April 2015. The majority of the apricot trees were hybrids under selection; one sample was collected from the cultivar Early Blush®Rutbhart (2928). Entire main branches with cankers were cut from trees when possible; otherwise, only the canker was taken. If exudates were present, they were also taken with the underlying tissues.

At room temperature, branches and cankers were washed, superficially disinfected (95° ethanol), rinsed with sterile water, and internal tissues at the margin between healthy and diseased tissues were collected. The weight of each sample was determined, then the samples were macerated in a stomacher (Bagmixer®, Interscience, St Nom la Bretèche, France) for 4 minutes in 30 ml of sterile phosphate buffer (8.75 g K₂HPO₄ and 6.75 g KH₂PO₄ in 1 L distilled water). Ten-fold dilutions of washings were then spread in Petri plates on two culture media: 10 % TSA with cycloheximide (3 g tryptone soy broth and 15 g of agar in 1 L of distilled water, 100 mg L⁻¹ cycloheximide) to assess of the total viable bacterial population. For the specific isolation of *P. syringae*, KBC medium (Mohan & Schaad, 1987) was used in this study. This medium was composed of 9 volumes of King's B medium (KB) with smaller quantity of proteose peptone n°3 than the original recipe (1.5 g K₂HPO₄, 1.5 g MgSO₄ 7H₂O, 15 g proteose-peptone n°3, 10 mL glycerol and 15 g agar in 900 mL distilled water), and 1 volume of boric acid (1.5 g H_3BO_3 in 100 mL distilled water). Moreover, it contained cycloheximide (100 mg L^{-1}) and cephalexin (80 mg L^{-1}). The culture media was incubated at 25°C for 48 - 72 hours. For each sample 30 putative P. syringae colonies based on morphological criteria of the colonies such as size, color, reflection of light, and roughness of the edge of the colonies were sub-cultured on KB medium. These colonies were further

This article is protected by copyright: All rights reserved. Parisi, L. (Auteur de correspondance), Morgaint, B., Blanco Garcia, J., Guilbaud, C., Chandeysson, C., Bourgeay, J.-F., Moronvalle, A., Brun, L., Brachet, M. L., Morris, C. E. (2019). Bacteria from four phylogroups of the Pseudomonas syringae complex can cause bacterial canker of apricot. Plant Pathology, 68 (7), 1249–1258. DOI: 10.1111/opa.13051 characterized as described below. This procedure has been adopted to take into account the polymorphism of colonies that we have observed in other work (Berge *et al.* 2014) and to avoid underestimating *P. syringae* population sizes. One strain of each colony type was stored in phosphate buffer and kept at 4°C. At this step of the process, the strains were not considered as pure culture strains, and could contain mixtures of *P. syringae* strains.

Strains from the CTIFL survey

These strains came from 26 orchards in 2011 (181 strains) and nine orchards in 2012 (68 strains). The orchards were mainly in Drôme and Ardèche counties and one orchard was in Isere County. The sampled cultivars were mainly Bergeron (660), Early Blush®Rutbhart (2928), Farbaly (4445) and Bergarouge®Avirine (2914), but Bergeval®Aviclo (3950), Farely (4587), Frisson (2821), Hargrand (1814), Jennycot, Orangé de Provence (1352), Primaris (4585), Tom Cot®Toyaco (2669) and Vertige (3845) were also sampled, sometimes only once. The sampled trees displayed clear canker symptoms, on main or small branches and on the trunk. In the majority of the samples, the symptomatic branch was cut and tissues at the margin between healthy and diseased tissues were analyzed. In some cases, bark and gum were sampled, in one case a fruit was analyzed. The samples were collected in individual bags, put in a cool box and transported in the CTIFL laboratories (Balandran, Gard county, or Etoile-sur-Rhône, Drôme county, following the location of the orchard), kept at 4°C and macerated at the latest 48h after the sampling. After this step, all the samples were frozen at -20°C and were processed in the same laboratory (CTIFL of Lanxade, Dordogne county), at the same time. A total of 249 strains were isolated (2 to 3 strains per sample) and they were identified by the presumptive morphology of the colonies on KBC medium (size, color, fluorescence under UV light), and by biochemical diagnostic tools (oxidase, aesculin and gelatin hydrolysis, levane tests) as members of the *P. syringae* complex (Bultreys & Kaluzna, 2010). These strains were stored in 20% glycerol (diluted in phosphate buffer), maintained at -80°C and sent to INRA, where they were analyzed with strains from UERI of Gotheron, as described below.

Characterization of bacterial strains for PG of P. syringae

A total of 779 strains were obtained and their phylogenetic context in the *P. syringae* complex was determined by PCR as described by Guilbaud *et al.* (2016), Borschinger *et al.* (2016) and Bartoli *et al.* (2014). Two multiplex PCRs allowed strains to be placed in PG01, 02, 03, 04, 09, 10 and 13 and a simplex PCR in PG07-08 (these two PG cannot be distinguished with this PCR), following the classification proposed by Berge *et al.* (2014).

Characterization of bacterial strains for hypersensitive reaction (HR) on tobacco, ice nucleation activity (INA) and production of syringomycin-like toxins.

A sub-set of 30 strains from PG01, 02, 03 and 07 was selected for the characterization for hypersensitive reaction in tobacco and ice nucleation activity (Table 1). The strains chosen for the characterization were representative of the PGs found in the PCR tests. Only the PG04 represented by a single isolate out of the 779 was not included in the characterization. From the 30 strains, 27 were isolated from symptomatic trees, mainly from cankers, in this study: 10 from experimental plots of the UERI experimental station and 17 strains from commercial orchards of the Drôme and Ardèche counties (France, Rhône-Alpes historical region). Three additional strains originated from our laboratory and were chosen as negative controls for pathogenicity to apricot and were included in this set of strains. Induction of a hypersensitive reaction (HR) in tobacco was performed as described by Morris *et al.*, (2007). Strains were also tested for ice nucleation activity (INA), following the protocol described by Berge et al (2014) : bacterial suspensions in phosphate buffer (10^7 CFU ml⁻¹) obtained from cultures growth on KB for three days at 25 °C were used to test INA. The bacterial suspensions were obtained by dilution of one pure bacterial colony in a 1 ml tube of sterile distillated water. Then the OD_{580} was adjusted to 0.06 to obtain a bacterial suspension of 10^8 CFU ml⁻¹, and 100µl of 10⁸ CFU ml⁻¹ suspension were diluted in 900 µl of sterile distillated water to obtain 1 ml at 10^7 CFU ml⁻¹. For each strain, three drops of 20 µl were deposited on an aluminum plate floating on a cooling bath. Freezing was determined at one degree intervals from - 2 °C to -15 °C. A strain was considered INA positive when at least two of the droplets froze at a temperature above - 9°C. Strains of PG02 were also tested for production of toxins based on a bioassay with Geotrichum candidum and the syringomycin medium (SRM) as described by Xu & Gross (1988) for detection of syringomycin-like toxins. Bacteria were grown for six days on SRM agar plates, then the plate was sprayed with a suspension of *G.* candidum at 10^8 fungal cells ml⁻¹. The inhibition zone around the bacterial colony was measured after two days as the distance between the edge of the fungus and the bacterial colony. The strain was considered as syringomycin-like positive when the inhibition zone was ≥ 1mm.

Characterization of pathogenicity on broad bean, melon, tomato and cherry fruits

Pathogenicity to apricot, and to three herbaceous species and cherry fruits was determined with a subset of 15 strains from this work and three control strains (not pathogen to apricot). In May 2016, seeds of Vicia faba (cv. Agua Dulce), Solanum lycopersicum (cv. Monalbo) and Cucumis melo (cv. Védrantais) were sown in the greenhouse. The seedbed was composed of fine blonde peat moss, sphagnum peat moss peat and coconut fiber. After 5 to 8 days, the plantlets were transplanted into 9 x 9 x 8 cm pots. The potting soil was composed of peat moss, sphagnum peat moss and pelletized clay. Then the plants were grown for approximatively 15 days under natural lighting. The temperature was between 12 and 34°C (mean temperature 21.2°C). The stems were then inoculated using three pinpricks at five cm interval and a drop of 10 μ l of a bacterial suspension in sterile distillated water $(10^7 CFUm)^{-1})$ was deposited on each wound; the negative control was inoculated with sterile water. The positive control was strain 41A, known to be pathogenic to broad bean, tomato and melon (Morris et al, 2019). For each strain, 4 plants were inoculated, providing 12 replicates. The plants were incubated in the greenhouse for 15 days before evaluation of symptoms: the length of external necrosis was measured, and the vascularization of the bacteria was evaluated by the length of internal necrosis observed after we made a longitudinal section of the plants. Immature cherry fruits of the cv. "Bigarreau géant d'Hedelfingen" were used for the test of pathogenicity on a subset of 12 strains. The protocol was described by Gross et al. (1984): 10 fruits were inoculated for each strain (three pricks per cherry) with a 10^6 CFU/ml suspension, and the fruits were incubated at 23.5 ± 1.5°C for a period of 120h. The length of necrosis was then evaluated. For all the tests, a strain was considered pathogenic when there was a significant difference ($p \le 0.05$) for the length of necrosis obtained compared to the water control with the non-parametric test of Kruskal-Wallis. Different level of aggressiveness were defined based on the differences of the length of necrosis by the same test.

Characterization of pathogenicity on apricot twigs

The positive control for pathogenicity to apricot was strain 41A, isolated in the CTIFL survey and used in the laboratory regularly for its aggressiveness in pathogenicity tests with apricot and other plants (Bartoli et al. 2015). Three strains from commercial French orchards, not pathogenic to apricot, were included in this test: P1.01.01.B03 and P3.01.09.C09, originating from asymptomatic P. armeniaca, and P4.01.01.C03, from a symptomatic P. domestica (Table1). The test was performed on dormant twigs of the cultivar "Bergeron" collected in winter, in commercial orchards. Twigs with a diameter of 8-15 mm (mean diameter of 11 mm for each experiment) were used. The twigs were cut in segments of 18 cm long, disinfected 1 min in sodium hypochlorite (1.3% active chlorine), rinsed 30 s in sterile water, then quickly shaken in 95°ethanol and rinsed. The cut twigs where then dried in a laminar flow hood for 20 min, and placed in transparent sterile plastic boxes (13 x 19 cm) with absorbent paper on the basal end of the twigs (five twigs per box). To avoid desiccation, the upper end was protected with Parafilm M laboratory film (Bemis North America, Neenah, Wisconsin, USA). A split of approximatively 1 cm was made with a cutter in the middle part of each twig, and then the twigs were inoculated with 20 μ l of a bacterial suspension in sterile distillated water (10⁸ CFUml⁻¹); two boxes and 10 twigs were prepared for each strain. The control was inoculated with sterile water. After inoculation, the boxes were humidified with 5 ml of sterile water poured on to absorbent paper placed under the twigs and stored at 18°C for 18 to 20 hours. Then, the boxes were incubated at $12^{\circ}C \pm 1^{\circ}C$ for 11 days, placed

This article is protected by copyright All rights reserved. Parisi, L. (Auteur de correspondance), Morgaint, B., Blanco Garcia, J., Guilbaud, C., Chandeysson, C., Bourgeay, J.-F., Moronvalle, A., Brun, L., Brachet, M. L., Morris, C. E. (2019). Bacteria from four phylogroups of the Pseudomonas syringae complex can cause bacterial canker of apricot. Plant Pathology, 68 (7), 1249–1258. DOI: 10.1111/ppa.13051 in a freezer at approximatively – 12°C for 42 h, and placed again at 12°C for 8 days. After 23 total days of incubation and freezing, the boxes were placed at 15°C \pm 1°C for 12 days. Then the bark was removed with a scalpel, and the length of superficial and internal necrosis was measured. A strain is considered pathogenic when there was a significant difference ($p \le 0.05$) for the length of internal lesion obtained in comparison to the negative control (water) with the non-parametric test of Kruskal-Wallis. This test does not permit to distinguish the different PGs of the *P. syringae* complex; the internal necrosis were similar for all PGs.

Phylogenetic analysis

A neighbour-joining phylogenetic tree of *P. syringae* was constructed on the basis of partial sequences of the citrate synthase housekeeping gene (*cts*) as previously described (Berge et al., 2014). Primers Cts-FP (forward): 5' AGT TGA TCA TCG AGG GCG CWG CC 3' and Cts-RP (reverse): 5' TGA TCG GTT TGA TCT CGC ACG G 3' were used for amplification and primer Cts-FS (fwd): 5'-CCC GTC GAG CTG CCA ATW TTG CTG A-3' was used for sequencing (Sarkar & Guttman, 2004). Analysis of partial *cts* gene sequences was performed as described previously using *P. syringae* strains for PGs, clades and pathovars identification as described by Berge *et al.* (2014) and Gomila *et al.* (2017), and the 30 strains from this study. Alignment of sequences was made by using DAMBE (version 5.3.70) and a neighbour joining tree was built with Mega (version 6.06).

Results

Apricot cankers harbor strains from *P. syringae* PG01, 02, 03, 04 and 07-08

The results concerning the situation of *P. syringae* strains from canker symptoms across the different PGs (PG01, 02, 03, 04, 07-08, 09, 10 and 13) are shown in table 2. The distribution of the strains in the different PGs was not different following their origin (Man Whitney U test, p-value=0.33). However, for the 530 strains collected in experimental orchards at UERI, the PGs found were mainly 1 (59.8%) and 2 (28.1%), followed by 3, 7-8 and 4. For the 249 strains collected in the CTIFL survey, we found mainly PG02 (69.5%), PG01 (21.7%), PG07-08 and PG03.

A wide diversity of strains can cause symptoms on apricot, independently of pathogenicity on herbaceous species and cherry fruits

The test of pathogenicity to apricot trees (Table 3) showed that 14 of the 15 strains isolated from symptomatic trees could cause superficial and internal necrosis to excised twigs and were considered as pathogenic; these strains belonged to PG01, 02, 03 and 07. One strain of PG07 (strain 3A) was not pathogenic to apricot and to broad bean, while all the other strains including the reference strains P1.01.01.B03, P3.01.09.C09 and P4.01.01.C03, not pathogenic to apricot, where able to induce symptoms to broad bean, with different aggressiveness levels. All the tested strains were pathogenic to melon and tomato, whatever their pathogenicity to apricot, also with different aggressiveness levels. The test of pathogenicity to cherries for a subset of 12 strains showed that eight strains were able to induce necrosis on the fruits, independently of their pathogenicity to apricots, broad bean, melon and

This article is protected by copyright All rights reserved. Parisi, L. (Auteur de correspondance), Morgaint, B., Blanco Garcia, J., Guilbaud, C., Chandeysson, C., Bourgeay, J.-F., Moronvalle, A., Brun, L., Brachet, M. L., Morris, C. E. (2019). Bacteria from four phylogroups of the Pseudomonas syringae complex can cause bacterial canker of apricot. Plant Pathology, 68 (7), 1249–1258. DOI: 10.1111/ppa.13051. tomato, HR induction on tobacco and ice nucleation activity. However, the strains that were avirulent to cherry belonged to PG01, 03 and 07, while the four PG02 tested strains were pathogenic and produced syringomycin-like toxins.

Phenotypes of strains from apricot cankers are different across PGs

From the 30 strains tested for the ability to induce HR on tobacco, 24 were HR⁺ (Table 3). However, this ability was linked to the PG of the strains: all the strains of PG02b, 02d and 03 were HR⁺, unlike the strains of PG07 that were HR⁻. In PG01a two of the eight strains tested did not induce hypersensitivity.

From the 30 strains tested for INA, 24 were INA^+ and 6 INA^- (Table 3). The great majority of strains of PG02 and PG07 were INA^+ (14/15 for PG02, 4/4 for PG07), but only half of strains of PG01a and 3 were INA^+ .

All the 16 strains of PG02b and 2d tested for toxin production on SRM produced syringomycin-like toxins (Table 3). The mean radius of inhibition was 8.12 ± 1.12 mm for PG02b strains and 6.00 ± 2.61 mm for PG02d.

Ability to induce hypersensivity in tobacco and ice nucleation activity are not adequate for predicting which strain are pathogenic to apricot

Table 4 shows the distribution of the 14 strains that were pathogenic to apricot trees across the two different phenotypes. Nine strains were INA^+ and HR^+ , showing that the pathogenicity to apricot is often associate with these two traits (Table 4). Seven of these

nine strains belonged to PG02b and 2d, one to PG01a and one to PG03. This result, taking into account that all the PG02 strains produce syringomycin-like toxins, show that a significant arsenal seems to be more frequent in this PG. However, two strains of PG07 HR⁻ were pathogenic to apricot, both INA⁺, and one PG 07 strain, harboring the same characteristics, was not pathogenic to apricot (table 3). Three strains, belonging to PG01a and PG03, were HR⁺ but INA⁻, showing that ice nucleation activity is not necessary to the pathogenicity to apricot.

Strains pathogenic to apricot are not necessarily closely related phylogenetically.

Here we compared strains isolated in this study with strains previously reported as pathogenic. The phylogenetic tree obtained with *cts* sequences is shown in figure 1. The *cts* gene was chosen as Berge *et al.* (2014) showed that its sequencing can accurately predict the phylogenetic affiliation of strains of the *P. syringae* complex. The tree showed that all the strains from PG 01 from this work belong to the clade 1a, and that strains that were pathogenic or not to apricot had identical *cts* sequences (P3.01.09.C09, 19B, 54B and P1.01.01.B03, 4A, 55D1, DG1a.21). Two of the strains that were pathogenic to apricot, 19B and 54B, grouped with four reference strains for the pathovars *maculicola, lachrymans, persicae* and *tomato*. In PG02b, the six strains studied had the same *cts* sequence as the reference strain for PG02b (type strain DSM10604^T), and close to the sequence of reference strain for *P. syringae* pv. *syringae* CRAFRU11. In PG02d, two strains grouped with pv. *aceris,* and the six other were clonal for *cts* sequences and close to the reference strain for pv. *syringae* B728a. In PG03, the two strains studied grouped with *P. syringae* pv. *morsprunorum* race 2, known to be pathogenic to apricot, but also with the pathovars *phaseolicola* and P.

mori. The four strains of PG07 studied belong to the PG07a, like *P. viridiflava*, and three of them were identical for *cts* sequences.

Discussion

In the literature, reports of bacteria causing canker on apricot suggest a limited diversity of strains as the causal agent: *P. syringae* pv. *syringae* (PG02) and *P. syringae* pv. *morsprunorum* race 1 (PG03) (Bultreys & Kaluzna, 2010). In this work, we isolated *P. syringae* from five different PGs from the internal tissues of diseased apricots: PG 01, 02, 03, 04, 07.

The high frequency of strains of PG 01 on symptomatic trees in apricot orchards is surprising, as only two bacteria of the *P. syringae* group are described in this PG for pathogenicity to stone fruits, and none on apricot: *P. syringae* pv. *morsprunorum* race 2 pathogenic to cherry and *P. syringae* pv. *persicae* pathogenic to peach (Bultreys & Kaluzna, 2010). The latter is an object of quarantine (A2 list of European Plant Protection Organization, EPPO, 2005), while *P. syringae* pv. *morsprunorum* is not concerned by quarantine. All the PG 01 strains found of this study belong to PG01a, and are phylogenetically close to pathovars never described as pathogenic to apricot.

Concerning PG02 (nearly 70% of the strains in commercial orchards), all the purified strains, whatever their origin, belong to clades 2b and 2d and were similar to strains of *P. syringae* pv. *syringae* known to be pathogenic to apricot (Lamichhane *et al.* 2014).

A small percentage of strains isolated from diseased apricot tissues belong to PG03; this PG contains *P. syringae* pv. *morsprunorum* race 1, pathogen to apricot tree, and *cts* sequencing confirmed the genetic proximity between these strains and this pathovar.

This article is protected by copyright All rights reserved. Parisi, L. (Auteur de correspondance), Morgaint, B., Blanco Garcia, J., Guilbaud, C., Chandeysson, C., Bourgeay, J.-F., Moronvalle, A., Brun, L., Brachet, M. L., Morris, C. E. (2019). Bacteria from four phylogroups of the Pseudomonas syringae complex can cause bacterial canker of apricot. Plant Pathology. 68 (7), 1249–1258, DOI: 10.1111/ppa.13051 Finally, only one isolate among the 779 characterized here belonged to PG 04. This PG is found on cropped and wild plants (mostly monocotyledonous), rain, snowpack and plant litter (Berge *et al*, 2014). The six strains from this PG described by Berge *et al*. (2014) were not pathogenic on cantaloupe indicator plants; however, this PG comprises seven pathovars. In other research work in commercial apricot orchards of Drôme county, 2'247 strains of *P. syringae* were sampled from different reservoirs (asymptomatic and symptomatic trees, leaf litter, and cover crop), but no PG04 strains were detected (Parisi, unpublished results). We hypothesized that this PG was not significantly involved in the etiology of bacterial canker in apricot orchards and we therefore focused our work on PG01, 02, 03 and 07.

Bacteria in PG07 were present in nearly 6% of the samples from both origins, belonging to PG07a, like *P. viridiflava*. However, *P. viridiflava*, pathogenic to a large range of plants (Bartoli *et al.* 2014) is not known to induce bacterial canker of stone fruits. On the other hand, *P. viridiflava* is known to cause blossom blight on woody plants such as kiwifruit and peach apoplexy (Lamichhane *et al.* 2014).

Obtaining strains from diseased tissues does not demonstrate the pathogenicity of these isolates, and therefore Koch's postulates must be verified. The test of pathogenicity to apricot trees showed that the great majority of the tested strains were pathogenic, confirming that members of *P. syringae* group of PG01, 02, 03 and 07 can induce apricot bacterial canker. This property was not described for bacteria of PG01 and 07.

The phenotypic tests made on a set of 30 strains were aimed to distinguish a putative common profile for the bacteria pathogenic to apricot; in fact, HR reaction on tobacco, ice nucleation activity and production of toxins are known to be linked to the pathogenicity of *P*. *syringae*. The results showed that there is a clear homogeneity for phenotypes of bacteria of

some PGs, . For example, nearly all the strains of PG02b and 2d (closely related to the pathovar *syringae*) are HR^+ , INA^+ and produce syringomycin-like toxins, like all the strains pathogenic to apricot in these two clades of PG02. This result is in agreement with the description of PG02 bacteria by Berge *et al.* (2014): this PG contains bacteria aggressive on cantaloupe seedlings, ice nucleation active and able to induce HR in tobacco, especially in the clades 2b and 2d. Syringomycin-like toxins are known to be produced by bacteria of this PG, as found in this work.

In the same manner, the four strains of PG07 are HR⁻. Bacteria in PG07 possess a noncanonical T3SS (Bartoli et al. 2014), and so don't always induce HR reaction. We demonstrate clearly in this work that P. viridiflava can induce bacterial canker in apricot; two strains were pathogenic in our tests, both HR-. The fact that diagnosis of diseases caused by bacteria often takes into account the HR reaction in tobacco could have contributed to the lack of identification of *P. viridiflava* as pathogenic in apricot, as HR- bacteria are sometimes eliminated before complete characterization (Giovanardi et al. 2018). It is interesting to note that if no peer-reviewed paper had previously reported the pathogenicity of *P. viridiflava* to apricot, French technical reports cite this bacteria as responsible for bacterial canker of apricot with *P. syringae* and *P. morsprunorum* (Edin et al. 2000). There are also two reports of isolation of P. viridiflava from apricots (without pathogenicity tests) in literature (Banapour, 1991, Harallah et al. 2004) and a report on the ability of P. viridiflava to establish an epiphytic phase on apricot (Bordjiba & Prunier, 1991). One of the two strains of PG03 pathogenic to apricot was HR^+ and INA^+ , but the other was HR^+ , INA^- . The same observation occurred in PG01a, only one strain pathogenic to apricot was HR⁺, INA⁺, the two other were HR^{+} , INA⁻. This demonstrate that pathogenicity to apricot does not necessitate the presence of ice nucleation activity and production of toxins, even if PG02b and PG02d

bacteria, the most abundant in commercial orchards in apricot tissues, display all these traits. We can also remark that this arsenal does not contain all the determinants of pathogenicity: strain P4.01.01C03, isolated on *Prunus domestica*, is not pathogenic to apricot.

Our results clearly demonstrate that genetic variability of the *P. syringae* strains pathogenic to apricot is much greater than previously reported, and that the pathogenicity of a strain cannot be predicted by the phylogenetic position or by the phenotypical characteristics for traits other than pathogenicity to apricot considered here. A rapid test with plants easy to grow in greenhouse is not possible to predict pathogenicity to apricot. The test on apricot shoots is long, time and space consuming, but it seems actually the only test available.

The results of this study call into question the notion of "pathovar"; very different bacteria from four PGs of *P. syringae* complex could have the same name referring to its pathogenicity to apricot tree. However, we demonstrate in greenhouse tests that these bacteria are also pathogenic on broad bean, melon and tomato, and that they display different phenotypic characters. The difficulty to characterize some pathovars of *P. syringae* was demonstrated and discussed by Morris *et al.* (2019) for strains from multiple origins.

An interesting question is to know if pathogenicity to apricot, found in very different genetic backgrounds in the *P. syringae* group, can be acquired by horizontal transfer, for example by transformation *in planta* as showed by Lowell *et al.* (2009)? This implies that great populations of bacteria can coexist in host plants, and that complex interactions between different strains can develop, as shown recently by Rufian *et al.* (2018). However, it is also possible that different mechanisms of host colonization and infection coexist in the *P. syringae* complex (Marceletti & Scortichini, 2018); genetic traits giving the ability to cause

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apricot disease might have emerged independently in bacterial reservoirs. P. syringae is a group of bacteria able to develop epiphytic phases, and that is present in many different substrates in agricultural and non-agricultural environments. In apricot orchards, reservoirs such as cover-crops or leaf litter are present along all the life of the tree. The question of the role of all these reservoirs in the emergence of pathogenic bacteria remains important for the implementation of measures of control of the disease, as cultural practices to reduce these reservoirs importance.

Another important question concerns the diagnostic tests that can be elaborated. Here we demonstrate that very different strains of the *P. syringae* complex, displaying different characteristics, must be detected for a good diagnostic. This is necessary for the detection of asymptomatic bacteria in plants such as young trees in nurseries, and this is also an important challenge if the apricot plant itself can be a reservoir, as demonstrated for cherries and P. morsprunorum (Dowler & Petersen, 1967). Producing healthy plants for young orchards plantation is the first step for an effective control of the disease.

The variability of *P. syringae* pathogenic to apricot has also to be taken into account in the selection of plants resistant to the disease, as for the other stone-fruits concerned about bacterial canker (Mgbechi-Ezeri et al. 2018).

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Legend

Figure1. Phylogenetic tree of *P. syringae* constructed on the basis of partial sequences of the citrate synthase housekeeping gene (*cts*). Analysis of partial *cts* gene sequences was performed using *P. syringae* strains for phylogroups (PG), clades (a, b, c, d, e) and pathovars (pv.) identification as described by Berge et al. (2014) and Gomila et al. (2017), and the 30 strains from this study. Alignment of sequences was made by using DAMBE (version 5.3.70) and a neighbour joining tree was built with Mega (version 6.06). Highlighted strains are those characterized in this work.

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PG and Clade	Strain	Year of isolation	Origin	French district and County	Isolated on	Kind of symptom
01a	4A	2011	CTIFL survey	Boucieu-le-Roi (07)	Sym. P. armeniaca	Trunk or main branch canker, f
01a	19B	2011	CTIFL survey	Buis-les-Baronnies (26)	Sym. P. armeniaca	Trunk or main branch canker, t
01a	54B	2011	CTIFL survey	Besignan(26)	Sym. P. armeniaca	Trunk or main branch canker,
01a	55D1	2011	CTIFL survey	Besignan(26)	Sym. P. armeniaca	Trunk or main branch canker,
01a	DG 1a.21	2015	UERI Gotheron	St Marcel-lès-Valence (26)	Sym. P.armeniaca	Trunk and main branch canker
01a	DG 12.20	2015	UERI Gotheron	St Marcel-lès-Valence (26)	Sym. P. armeniaca	Old cankers on main branch
01a	P1.01.01.B03	2014	Commercial orchard	Beauvoisin (26)	As. P. armeniaca	Buds
01a	P3.01.09.C09	2014	Commercial orchard	Larnage (26)	As. P. armeniaca	Branch tissues
02b	11C	2011	CTIFL survey	Vernoux-en-Vivarais (07)	Sym. P. armeniaca	Trunk or main branch canker,
02b	26D	2011	CTIFL survey	Bren (26)	Sym. P. armeniaca	Trunk or main branch canker,
02b	41A	2011	CTIFL survey	Larnage (26)	Sym. P. armeniaca	Main branch canker
02b	41D	2011	CTIFL survey	Larnage (26)	Sym. P. armeniaca	Trunk or main branch canker,
02b	53B	2011	CTIFL survey	Marsaz (26)	Sym. P. armeniaca	Trunk or main branch canker,
02b	DG 1a.7	2015	UERI Gotheron	St Marcel-lès-Valence (26)	Sym. P. armeniaca	Trunk or main branch canker

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02b	DG 7.8	2015	UERI Gotheron	St Marcel-lès-Valence (26)	Sym. P. armeniaca	Trunk and main branch car
02b	P4.01.01C03	2014	Commercial orchard	Mollans-sur-Ouvèze (26)	Sym. P. domestica	Trunk canker
02d	18	2011	CTIFL survey	Colombier-le-Jeune (07)	Sym. P. armeniaca	Trunk or main branch cank
02d	7C	2011	CTIFL survey	Gilhoc-sur-Ormèze (07)	Sym. P. armeniaca	Trunk or main branch cank
02d	37C	2011	CTIFL survey	Etoile-sur-Rhône (26)	Sym. P. armeniaca	Trunk or main branch cank
02d	39D	2011	CTIFL survey	Etoile-sur-Rhône (26)	Sym. P. armeniaca	Trunk or main branch cank
02d	71B	2011	CTIFL survey	Plaisians (26)	Sym. P. armeniaca	Trunk or main branch cank
02d	DG 10.14	2015	UERI Gotheron	St Marcel-lès-Valence (26)	Sym. P. armeniaca	Main branch canker
02d	DG 12.6	2015	UERI Gotheron	St Marcel-lès-Valence (26)	Sym. P. armeniaca	Old cankers on main branc
02d	DG 14.7	2015	UERI Gotheron	St Marcel-lès-Valence (26)	Sym. P. armeniaca	Main branch canker
03	93A	2011	CTIFL survey	Gilhoc-sur-Ormèze (07)	Sym. P. armeniaca	Trunk or main branch cank
03	DG 1b.9	2015	UERI Gotheron	St Marcel-lès-Valence (26)	Sym. P. armeniaca	Trunk or main branch cank
07	3A	2011	CTIFL survey	Vaudevant (07)	Sym. P. armeniaca	Trunk or main branch cank
07	93D	2011	CTIFL survey	Gilhoc-sur-Ormèze (07)	Sym. P. armeniaca	Trunk or main branch cank
07	DG 8.15	2015	UERI Gotheron	St Marcel-lès-Valence (26)	Sym. P. armeniaca	Old canker on branch
07	DG 9.13	2015	UERI Gotheron	St Marcel-lès-Valence (26)	Sym. P. armeniaca	Branch and main branch ca

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Origin	No. orchards	No. trees	No. strains	PG 01	PG 02	PG 03	PG 04	PG 07- 08	PG 09	PG 10	PG 13	PG Ni
UERI	8	15	530	59.8	28.1	5.6	0.2	5.6	0	0	0	0.6
CTIFL survey	55	Nk	249	21.7	69.5	0.4	0	6	0	0	0	2.4

Table 2. Distribution (%) in eight phylogroups of the *P. syringae* species complex of 779 strains isolated from diseased tissues of apricot in French orchards. Nk = not known. Ni = not identified.

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					Pathoge	nicity to			
PG and			SRM	INA		Broad			
Clade	Strain	HR	(mm)	(°C)	Apricot	bean	Melon	Tomato	Cherry
1a	4A			-2.6					
1a	19B				P+	P ++	P ++++	P ++	P +
1a	54B			-2.6	_				
1a	55D1			-2.2	P +	P +++	P ++++	P ++	
1a	DG 1a.21				P +	P +++	P ++++	P ++	P +
1a	DG12.20			-2.3					
1a	P1.01.01.B03				P -	P ++	P +++	P ++	Ρ-
1a	P3.01.09.C09				P -	P +	P ++	P ++	Ρ-
2b	11C		6	-1.8					
2b	26D		_7	-2.3					
2b	41A		8	-1.3	P+	P ++	P +++	P ++	P +
2b	41D		9	-1.9	P+	P +++	P +++	P ++++	P +
2b	53B		8						
2b	DG 1a.7		9	-5	P+	P +++	P +++	P +	
2b	DG 7.8		9	-2.6	P+	P +	P +++	P ++	
2b	P4.01.01C03		9	-2.3	P -	P +++	P +	P +	P +
2d	1B		5	-3.2	P+	P ++++	P ++++	P ++	P ++
2d	7C		_5	-3.4	P+	P ++++	P ++++	P +++	P ++
2d	37C		4	-1.5					
2d	39D		3	-2.1					
2d	71B		6	-1.4					
2d	DG 10.14		10	-2.1					
2d	DG 12.6		10	-2.2	P+	P +++	P ++	P +++	
2d	DG 14.7		5	-2.2					
3	93A			-3.8	P+	P ++++	P +++	P +++	

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	7 7 7	93 D0 D0
postprin	Table 3. Ph on tobacco	ien , ic

e d t e

3

DG 1b.9			P +	P +	P +	P +	Ρ-
3A		-3.1	Ρ-	Ρ-	P +	P +	Ρ-
93D		-2	P+	P +	P +++	P ++	
DG 8.15		-1.9	P+	P +++	P +++	P ++	P +
DG 9.13		-2.5					

otypic characterization of 30 strains of *P. syringae* complex : hypersensitive reaction (HR) e nucleation activity (INA), toxins production in syringomycin-like medium (SRM) of PG02 strains; pathogenicity to apricot, broad bean, melon (18 strains), and pathogenicity to cherry (12 strains). INA is positive when INA T°C \geq -9 °C. SRM: radius of inhibition zone (mm). Positive reaction. Negative reaction. Not tested.

P-: not pathogenic. P+ to P+++: pathogenic with increasing level of aggressiveness

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HR	INA	PG 01a	PG 02b	PG 02d	PG 03	PG 07	Total		
Positive	Positive	1	4	3	1	0	9		
Positive	Negative	2	0	0	1	0	3		
Negative	Positive	0	0	0	0	2	2		
Total		3	4	3	2	2	14		
Table 4 : ability to induce hypersensitive reaction (HR) on tobacco and ice nucleation activity (INA)									
of 14 strain	s of the <i>P. syri</i>	<i>ingae</i> complex	pathogenic t	o apricot.					

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