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Pathogen profile

Xanthomonas arboricola pv. *pruni*, causal agent of bacterial spot of stone fruits and almond: its genomic and phenotypic characteristics in the *X. arboricola* species context

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SUMMARY

BACKGROUND: *Xanthomonas arboricola* pv. *pruni* (*Xap*) causes bacterial spot of stone fruits and almond, an important disease that may reduce the yield and vigour of the trees, as well as the marketability of affected fruits. *Xap* lies within the *Xanthomonas* genus, which has been intensively studied because of its strain specialization and host range complexity. Here, we summarize the recent advances in our understanding of the complexities of *Xap*, including studies of the molecular features that result after comparative phenotypic and genomic analyses, in order to obtain a clearer overview of the bacterial behaviour and infection mechanism in the context of the *X. arboricola* species.

TAXONOMIC STATUS: Bacteria; Phylum *Proteobacteria*; Class *Gammaproteobacteria*; Order *Xanthomonadales*; Family *Xanthomonadaceae*; Genus *Xanthomonas*; Species *X. arboricola*; Pathovar *pruni*.

HOST RANGE AND SYMPTOMS: *Xap* infects most *Prunus* species, including apricot, peach, nectarine, plum and almond, and occasionally cherry. Symptoms are found on leaves, fruits, twigs and branches or trunks. In severe infections, defoliation and fruit dropping may occur.

DISTRIBUTION: Bacterial spot of stone fruits and almond is worldwide in distribution, with *Xap* being isolated in Africa, North and South America, Asia, Europe and Oceania. It is a common disease in geographical areas in which stone fruits and almonds are grown. *Xap* is listed as a quarantine organism in several areas of the world.

GENOME: The genomes of six isolates from *Xap* have been publicly released. The genome consists of a single chromosome of around 5 000 000 bp with 65 mol% GC content and an extrachromosomal plasmid element of around 41 000 bp with 62 mol% GC content. Genomic comparative studies in *X. arboricola* have allowed the identification of putative virulence components associated with the infection process of bacterial spot of stone fruits and almond.

DISEASE CONTROL: Management of bacterial spot of stone fruits and almond is based on an integrated approach that comprises essential measures to avoid *Xap* introduction in a production zone, as well as the use of tolerant or resistant plant material and chemical treatments, mainly based on copper compounds. Management programmes also include the use of appropriate cultivation practices when the disease is already established. Finally, for the effective control of the disease, appropriate detection and characterization methods are needed for use in symptomatic or asymptomatic samples as a first approach for pathogen exclusion.

USEFUL WEBSITES: https://gd.eppo.int/taxon/XANTPR; http://www.cost.eu/COST_Actions/ca/CA16107; http://www.xanthomonas.org

Keywords: bacterial spot, *Prunus*, *Xanthomonas*.

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XANTHOMONAS ARBORICOLA: CAUSAL AGENT OF PLANT DISEASES, BUT NOT ONLY THAT

Xanthomonas arboricola is essentially a bacterial complex predominantly associated with crop diseases in a wide range of plant hosts (Lamichhane, 2014). Strains of *X. arboricola* species consist of a bacterial group with a high host specialization. Most of the strains of the species have been identified to be pathogenic in herbaceous or woody plants, but, in the last few years, other strains, e.g. in the pathovars *celebensis*, *fragariae* and *populi*, and other non-pathovar-assigned strains, have been considered to be non-pathogenic, low-virulent or opportunistic pathogens (Essakhi *et al.*, 2015). Moreover, some of these non-pathogenic xanthomonads strains have been found to cohabit with pathogenic strains on the same host plant (Cesbron *et al.*, 2015; Garita-Cambronero *et al.*, 2016a,b). The existence of pathogenic and non-pathogenic strains phylogenetically close, but drastically different in their phenotype, makes *X. arboricola* a useful model to perform

comparative microbe–plant interaction studies in order to reveal the pathogenicity and host range mechanisms.

Xanthomonas arboricola pv. *arracaciae*, *X. arboricola* pv. *celebensis*, *X. arboricola* pv. *corylina*, *X. arboricola* pv. *fragariae*, *X. arboricola* pv. *guizotiae*, *X. arboricola* pv. *juglandis*, *X. arboricola* pv. *populi*, *X. arboricola* pv. *pruni* and *X. arboricola* pv. *zantesdeschiae* are the nine pathovars proposed according to the host range of the species (Fischer-Le Saux *et al.*, 2015). Among these pathovars, *corylina*, *juglandis* and *pruni* are considered to be the most virulent and economically important. Indeed, pathovars *corylina* and *pruni* are classified as quarantine pests in many countries, for instance in the European Union (EFSA PLH Panel, 2014; OJEC, 2000; Fischer-Le Saux *et al.*, 2015). With regard to the remaining six pathovars of *X. arboricola*, all have been defined as composed of "poorly pathogenic strains" or "saprophytic or opportunistic pathogens", not being responsible for pandemics in a defined host range (Fischer-Le Saux *et al.*, 2015; Haworth and Spiers, 1992; Hayward, 1993; Lamichhane, 2014; Vandroemme *et al.*, 2013a).

The disease symptoms produced by the pathovars *corylina*, *juglandis* and *pruni* have been characterized, as infected plants show angular necrotic spots and cankers on the leaves, branches and fruits, and, in severe infections, the trees may be weakened, being less productive. In all cases, the diseased fruits are generally commercially unacceptable because of their symptoms which make them unmarketable. Intuitively, infections produced by these pathogens in their respective hosts appear comparable and yield similar damage. The substantive differences amongst these three most virulent pathovars are the specific adaptations of the bacteria to the host to create an optimum environment for their survival and proliferation: for instance, by specific mobilization of nutrients or by blocking the plant defence mechanisms developed to prevent infection. Host specificity is the result of different adaptive mechanisms that involve all stages of the infection process, starting with the first contact of the bacterium with the plant, and continuing with proliferation inside the tissues (Jacques *et al.*, 2016). The breakdown in resistance associated with the host is, in various xanthomonads, a result of the inactivation of avirulence genes or suppression of pattern-triggered (PTI) and effector-triggered (ETI) immunity (Bigeard *et al.*, 2015; Jacques *et al.*, 2016). In *X. arboricola*, the suppression of plant immunity has been demonstrated to be correlated with the host range according to the repertoires of type III effectors (T3Es) of the type III secretion systems (T3SSs) (Jacques *et al.*, 2016).

Xanthomonas arboricola encompasses bacterial strains that cannot be assigned to any of the existing pathovars, although sometimes were initially wrongly assigned to an incorrect pathovar (Cesbron *et al.*, 2015; Garita-Cambronero *et al.*, 2017). These bacteria may provide a source for the exchange of genetic material and may be involved in the evolution of pathogenic lineages (Jacques *et al.*, 2016). Although the three major pathovars, *pruni*, *corylina* and *juglandis*, are well defined by three clonal complexes with a common ancestor, the other strains of the species, either weakly or not pathogenic, are genetically and phylogenetically heterogeneous.

Amongst the major pathogens, pathovar *pruni* is the most monomorphic, being highly adapted to *Prunus* spp. with a prominent pathogenicity, and showing a low genetic variation amongst strains (Fischer-Le Saux *et al.*, 2015).

DISTRIBUTION AND ECONOMIC IMPACT OF BACTERIAL SPOT OF STONE FRUITS AND ALMOND

Xanthomonas arboricola pv. *pruni* (*Xap*) is a pathogen highly specialized to infect *Prunus* species and is responsible for important economic losses in this crop worldwide (Stefani, 2010). *Xap* is currently present in the five continents, in almost all countries with stone fruit production (EFSA PLH Panel, 2014). The distribution of bacterial spot disease is not homogeneous, being considered widespread in some countries, whereas, in others, it is under eradication and only local and sporadic outbreaks have been registered. Surprisingly, reports of *Xap* on almond and, particularly, on cherry are scarce (Jami *et al.*, 2005; Palacio-Bielsa *et al.*, 2010). International trade has led to the long-distance dissemination of *Xap*, mainly through latently infected plant material used for propagation (Goodman and Hatting, 1986; López-Soriano *et al.*, 2016). At short distance, rain, wind and contaminated pruning tools or machinery can spread *Xap* amongst trees and nearby plots (Goodman, 1988). The disease is more frequent and severe in areas with a temperate and humid climate. Warm temperatures (19–28 °C) and high humidity favour the multiplication of the bacterium (Morales *et al.*, 2017).

The economic impact of the disease is a result of the reduced quality and marketability of fruits, reduced orchard productivity and increased costs of nursery production (Stefani, 2010). There is scarce information about the real cost associated with disease outbreaks. In the USA, 25%–75% of fruits showed lesions in neglected peach orchards and, in South America, the presence of *Xap* caused severe losses and limited the production of susceptible cultivars of peach and nectarine (Palacio-Bielsa *et al.*, 2015; Stefani, 2010). With regard to Europe, it has been estimated that a hypothetical outbreak of the disease in a commercial plum orchard in the Emilia Romagna area (northern Italy), affecting 30% of fruits, could result in crop losses of over 9500 or 11 200€/ha (Stefani, 2010). In Spain, yield losses in commercial almond orchards in northern Spain ranged between 23% and 47% of production in 2013 and 2014 (Palacio-Bielsa *et al.*, 2015). Currently, the presence of *Xap* is also a problem for ornamental plant nurseries, mainly for

those producing cherry laurel in the Netherlands destined for international trade (Tjou-Tam-Sin *et al.*, 2012).

XANTHOMONAS ARBORICOLA PV. *PRUNI*: AN EXAMPLE OF HOST ADAPTATION AND NO SPECIFIC SYMPTOMATOLOGY

The natural hosts of *Xap* are *Prunus* species, especially cultivated fruit crops and their hybrids, such as European and Japanese plum, peach and nectarine, apricot, almond, and sweet and sour cherry. Other hosts are several ornamental and spontaneous *Prunus* species, such as Japanese apricot, Chinese wild peach (*P. davidiana*), cherry laurel, *P. buergeriana*, *P. crassipes* and *P. donarium* (EFSA PLH Panel, 2014).

Studies to elucidate the factors that contribute to the *Xap* host range, and the different stages in the infection process, have revealed differences between *Xap* and the other *X. arboricola* pathovars, and between *Xap* and the other *Xanthomonas* spp. that infect *Prunus* spp. Dissimilarity was found in the content of genes involved in the initial mechanisms of infection and in the virulence elements that participate in the creation of a favourable environment for the bacterial colonization and infection process (Cesbron *et al.*, 2015; Garita-Cambronero *et al.*, 2017; Jacques *et al.*, 2016). The T3SS has been revealed as being particularly important in the virulence of *Xap*, being one of the pathovars with the largest effector repertoire, which is in accordance with its virulence (Lamichhane, 2014).

With regard to the symptoms of bacterial spot, these can be observed on the leaves, fruits, twigs and branches, but no symptoms occur in flowers. Although some differences may be observed, symptoms have common features in all *Prunus* species (EFSA PLH Panel, 2014; Roselló *et al.*, 2012). At the early stages, infection in leaves is revealed by the appearance of small translucent or pale green-coloured spots, which evolve to dark or black necrosis and enlarge through time. These lesions are polygonal, delimited by the side ribs, visible on both sides of the leaves and are often clustered in areas of water accumulation, such as the central nerve and the apical edge of the leaf (Fig. 1A). In peach and nectarine (*P. persica* and *P. persica* var. *nucipersica*, respectively), a characteristic gradient of colour (brown to yellow to green) can be observed in the apical part of some leaves (Fig. 1B) and, when they are severely affected, they turn yellow and drop. In almond (*P. amygdalus*, syn. *P. dulcis*), yellowing of the leaves and defoliation are rarely observed (Palacio-Bielsa *et al.*, 2010; Roselló *et al.*, 2012). In European and Japanese plum (*P. domestica* and *P. salicina*, respectively), sweet and sour cherry (*P. avium* and *P. cerasus*, respectively) and cherry laurel (*P. laurocerasus*), leaves remain on the tree and acquire a shot-hole appearance on detachment of dried tissues (Fig. 1C).

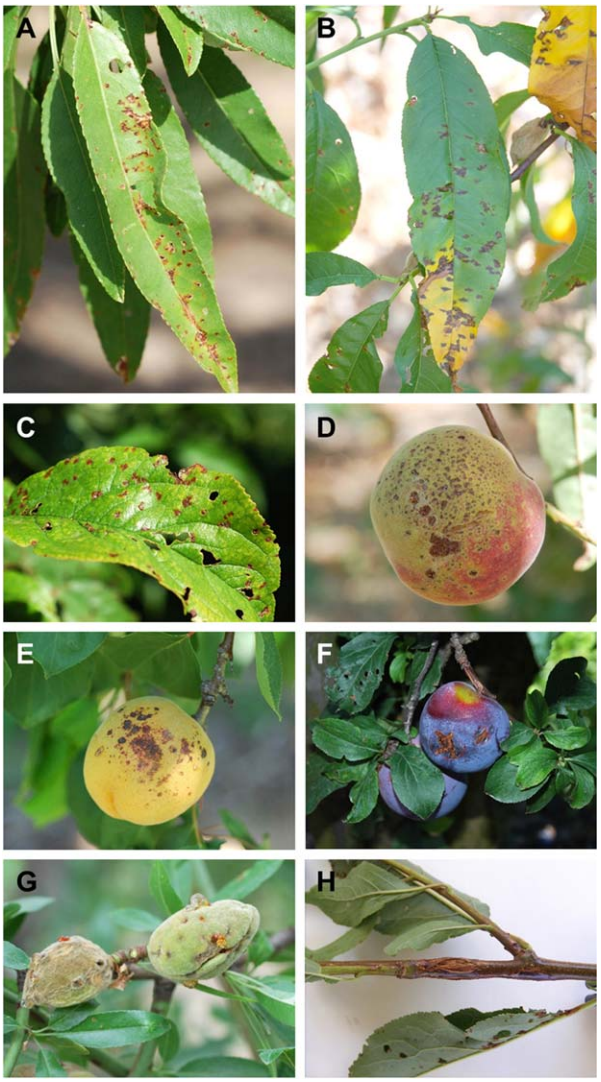

Fig. 1 Symptoms of *Xanthomonas arboricola* pv. *pruni* on *Prunus* spp. (A) Leaf spot clustering on almond leaves. (B) Leaf spots on peach, followed by yellowing and browning. (C) Shot-hole appearance on plum leaf. Characteristic lesions on peach (D), apricot (E) and plum (F) fruits. (G) Initial sunken and corky almond nut lesions (right), and latter raised lesions on dehydrated mesocarp (left). (H) Characteristic branch canker on plum. Photographs courtesy of M. A. Cambra.

In peach, nectarine, apricot (*P. armeniaca*) and plum fruits, small brown depressed spots surrounded by a yellowish-green halo can be observed. As the fruits grow, the spots become necrotic and can be grouped, taking irregular shapes. Pitting and cracking may occur, and sometimes gummy exudates appear (Fig. 1D–F). Symptoms on almond are very characteristic. During spring, sunken, corky lesions, oozing gum that streams or clumps, can be observed. The sunken lesions become raised following dehydration of the mesocarp in summer, and these areas remain attached to the endocarp preventing the natural peeling of the

fruits (Fig. 1G). In some cases, dark spots are observed on the endocarp, which can affect the nut. Infected fruits either drop prematurely or remain on the tree after harvest (Palacio-Bielsa *et al.*, 2010; Roselló *et al.*, 2012).

Damage to the branches and trunk is not as frequent as in the leaves and fruits. Canker symptoms occur on plum trees, but are rare on peach and almond trees (Battilani *et al.*, 1999; Roselló *et al.*, 2012; Stefani, 2010). When lesions appear in twigs, they are dark and elongated, slightly depressed and often have a shiny, greasy appearance and water-soaked margins. If lesions expand, they can girdle the twig and cause tip dieback. Early twig infection can lead to canker in late summer. Unlike the cankers on peach, those on plum are perennial and develop continuously on young twigs, and can lead to the formation of large cankers affecting the branches and trunk (Fig. 1H). In severe infections, twigs may be strangled and cause tip dieback, leading to defoliation, fruit drop and plant debilitation (Lamichhane, 2014).

Symptoms of bacterial spot disease can sometimes be confused with injuries caused by other bacteria, fungi, viruses or nutrient deficiencies, or abiotic factors such as wounds caused by wind or cultural practices (Lamichhane, 2014; Roselló *et al.*, 2012). Therefore, a diagnostic analysis is required to confirm the presence of *Xap* in order to take appropriate management decisions.

TAXONOMY AND CHARACTERIZATION OF THE CAUSAL AGENT OF BACTERIAL SPOT OF STONE FRUITS AND ALMOND

Xanthomonas arboricola pv. *pruni* (Vauterin *et al.*, 1995) (*Gammaproteobacteria*, *Xanthomonadales*, *Xanthomonadaceae*) is a Gram-negative, rod-shaped, monoflagellated, motile, non-sporulating aerobic bacterium. According to the reactions in the Biolog GN2 system, strains from this pathovar are able to metabolize α-D-glucose, α-ketoglutaric acid, bromosuccinic acid, D-cellobiose, D-fructose, D-mannose, D-psicose, D-trehalose, dextrin, glycyl-L-glutamic acid, L-glutamic acid, L-proline, L-serine, pyruvic acid methyl ester, succinic acid, succinic acid monomethyl ester, sucrose and Tween 20 (Garita-Cambronero *et al.*, 2016b,c).

Multilocus sequence analyses (MLSAs), based on the genes *dnaK*, *fyuA*, *gyrB* and *rpoD* (Garita-Cambronero *et al.*, 2017; Young *et al.*, 2008), or on the partial sequences of *atpD*, *efp* and *glnA* (Boudon *et al.*, 2005; Fischer-Le Saux *et al.*, 2015), have been useful in differentiating the pathovar *pruni* from the other pathovars of the species, confirming its phylogenetic proximity to the pathovars *corylina* and *juglandis*. These studies have concluded that these pathovars form monophyletic groups with a low genetic diversity amongst their strains, and must be considered as clonal complexes. Genetic variability amongst strains of *Xap* was also assessed by integron gene cassette array, as well as with BOX-PCR and repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) (Barionovi and Scortichini, 2006; Kawaguchi, 2014). Low genetic variability amongst *Xap* strains was shown, although the study indicated that integrons played a role in configuring the genetic diversity of this species. Despite this, none of the patterns observed in *Xap* were associated with any of the host plants or the geographical region from which the strains were isolated.

Moreover, new studies based on whole-genome analysis, using either concatenated sequences of the core group of genes of *X. arboricola* (Cesbron *et al.*, 2015; Garita-Cambronero *et al.*, 2016b,c) or pangenomic analysis of at least 7074 protein coding sequences, established pathovar *pruni* as a monophyletic group; however, in contrast with the observations of MLSA, slight differences were observed in strains from different geographical origins. Despite this, the average nucleotide identity amongst all members of this pathovar was over 99% (Garita-Cambronero *et al.*, 2017) (Fig. 2; Table 1).

Recently, a multilocus variable number of tandem repeats (MLVA) scheme has determined that at least 23 loci are reliable for the assessment of the genetic variability at the intrapathovar level, even at small spatiotemporal scales (Bergsma-Vlami *et al.*, 2012; Cesbron *et al.*, 2014; López-Soriano *et al.*, 2016). This approach provided the ability to classify 239 strains of *Xap*, recorded in the early 2000s in Spain, into 119 different MLVA haplotypes subdivided into 18 genetic clusters. Furthermore, the 25 *Xap* strains from a world collection analysed in this work were classified into 23 haplotypes, meaning that almost every strain formed a unique haplotype. At the same time, the Spanish strains were clearly separated from those of the worldwide collection (López-Soriano *et al.*, 2016). Therefore, MLVA showed a high discriminatory power in *Xap* to the extent that determined the potential multiple introduction of this pathogen in Spain.

After a phenotypic and genotypic characterization of a representative group of *Xanthomonas* strains associated with *Prunus* from Spanish outbreaks of bacterial spot, some *Xanthomonas*-like colonies were not clustered with *Xap* according to the MLSA scheme mentioned above (Garita-Cambronero *et al.*, 2016b,c, 2017). Moreover, three strains isolated from nectarine have been proposed recently as potential novel species of *Xanthomonas* associated with bacterial spot in nectarine (López-Soriano, 2017). In addition, another seven strains were identified as members of *X. arboricola*, but could not be placed within any of the nine pathovars described for this species (Garita-Cambronero *et al.*, 2017). Interestingly, these "atypical" strains have been found not only in *Prunus*, but also in other hosts, such as *Juglans* spp., where they have been described as sharing the habitat with strains of *X. arboricola* pv. *juglandis* (Cesbron *et al.*, 2015; Jacques *et al.*, 2016). In *Prunus*, these strains differed from *Xap* in several phenotypic characters, such as the swarming motility pattern (dendritic or circular colony), the utilization of carbon compounds, the chemotactic activity and, primarily, in their ability to cause disease on

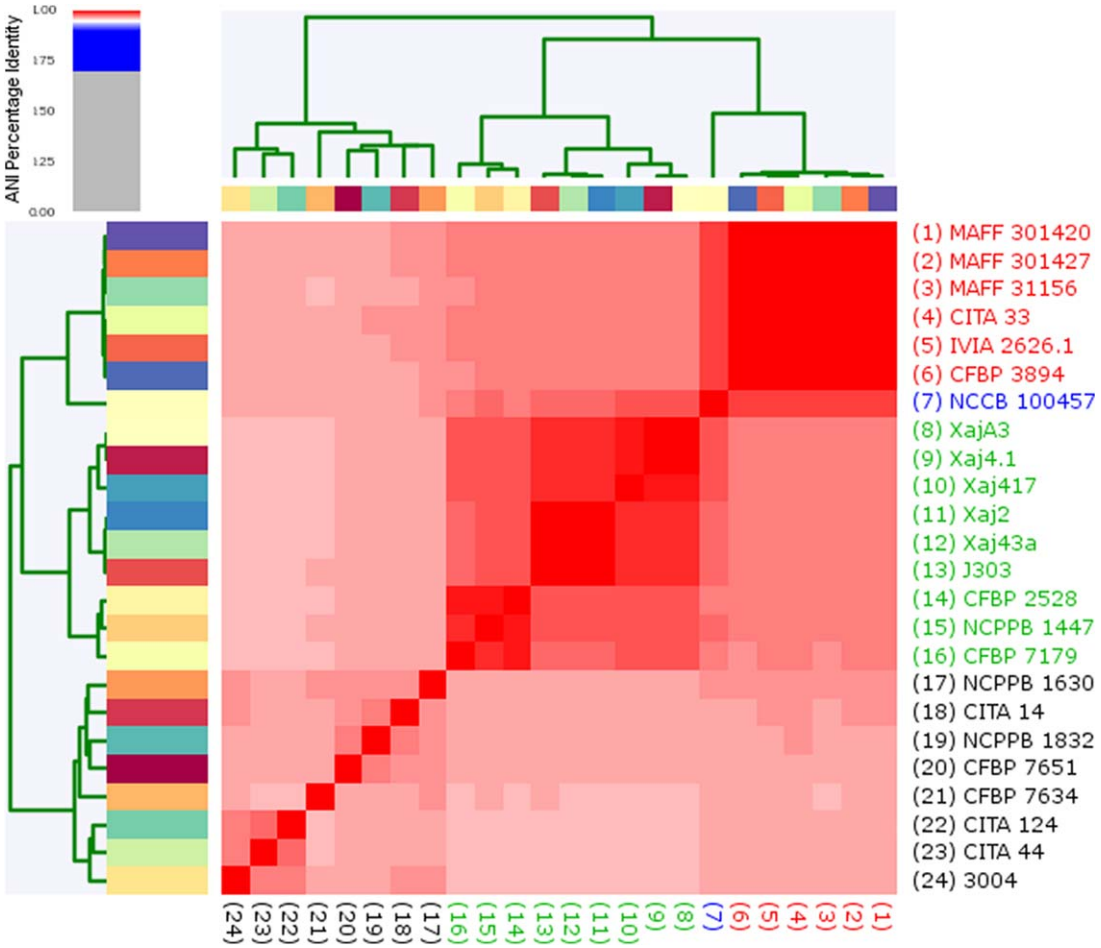

Fig. 2 Average nucleotide identity (ANI) amongst the whole genomes from strains of pathogenic [*Xanthomonas arboricola* pv. *corylina* (blue), *X. arboricola* pv. *juglandis* (green) and *X. arboricola* pv. *pruni* (red)] and low pathogenic or non-pathogenic strains of *X. arboricola* (black).

almond, apricot, peach and plum (Garita-Cambronero *et al.*, 2016b,c).

COMPARATIVE GENOMICS AND DESCRIPTION OF THE VIRULENCE FACTORS PRESENT IN *XAP*

Whole-genome comparative analysis within *X. arboricola* species has revealed not only that these *Prunus* non-pathogenic strains cannot be clustered within the pathovar *pruni*, but also that they are phylogenetically related to a heterogeneous group of non-pathogenic or low-virulent strains isolated from a wide host range, such as banana, barley and walnut (Garita-Cambronero *et al.*, 2017) (Fig. 2).

In addition to these phylogenetic analyses, initial descriptions of these genomes have permitted the ability to find variation amongst the pathogenic features presented in this low-virulent group and those members of the most virulent pathovars. Most showed remarkable variants in genomic regions associated with the regulation and structure of the hrc/hrp T3SS and related T3Es (Essakhi *et al.*, 2015; Ignatov *et al.*, 2015). In the light of evidence of the existence of pathogenic and non-pathogenic strains of *X. arboricola*, apparently evolving in sympatry on *Prunus* spp., potential virulence factors associated with the development of bacterial spot disease have been identified (Cesbron *et al.*, 2015; Garita-Cambronero *et al.*, 2016b,c, 2017).

The public availability of the genome sequence of *X. arboricola* pv. *juglandis* NCPPB 1447 in 2012 marked the beginning of the genomics era for *X. arboricola*. Since then, the efforts to generate genomic data for this species have increased significantly. To date, genome sequences of 29 strains have been obtained (Cesbron *et al.*, 2015; Garita-Cambronero *et al.*, 2014, 2016a,b,c, 2017; Gétaz *et al.*, 2018; Harrison *et al.*, 2016; Higuera *et al.*, 2015; Ibarra Caballero *et al.*, 2013; Ignatov *et al.*, 2015; López-Soriano *et al.*, 2016), as well as the whole sequence of the plasmid pXap41 of *Xap* (Pothier *et al.*, 2011) (Table 1).

Table 1 Statistics of the publicly available whole-genome sequence projects of *Xanthomonas arboricola*.

	Strain	GenBank accession	Size (Mb)	GC%	Genes	Proteins
Xanthomonas arboricola	CITA 14	LXIB01	4.86	65.6	4061	3870
Xanthomonas arboricola	CITA 44	LJGM01	4.76	65.8	4002	3728
Xanthomonas arboricola	CITA 124	LXKK01	4.75	65.8	4086	3798
Xanthomonas arboricola	CFBP 7634	JZEH01	4.93	65.6	4110	4006
Xanthomonas arboricola	CFBP 7651	JZEI01	5.03	65.5	4191	4086
Xanthomonas arboricola	3004	AZQY01	4.76	66.0	3997	3775
Xanthomonas arboricola pv. *celebensis*	NCPPB 1630	JPHE01	4.98	65.5	4113	3977
Xanthomonas arboricola pv. *celebensis*	NCPPB 1832	JPHC01	4.90	65.6	4081	3967
Xanthomonas arboricola pv. *corylina*	NCCB 100457	APMC02	5.22	65.5	4365	4089
Xanthomonas arboricola pv. *juglandis*	Xaj 417	CP012251.1	5.21	65.4	4358	4188
Xanthomonas arboricola pv. *juglandis*	J303	LSGZ01	5.06	65.5	4262	4040
Xanthomonas arboricola pv. *juglandis*	CFBP 2528	JZEF01	5.08	65.5	4263	4095
Xanthomonas arboricola pv. *juglandis*	CFBP 7179	JZEG01	5.15	65.4	4351	4194
Xanthomonas arboricola pv. *juglandis*	NCPPB 1447	AJTL01	5.02	65.4	4308	3921
Xanthomonas arboricola pv. *juglandis*	Xaj2	LHBK01	5.10	65.4	4266	4081
Xanthomonas arboricola pv. *juglandis*	Xaj43a	LHBT01	5.14	65.4	4314	4115
Xanthomonas arboricola pv. *juglandis*	XajA3	LHBS01	5.11	65.6	4283	4093
Xanthomonas arboricola pv. *juglandis*	Xaj4.1	LHBL01	5.11	65.6	4285	4102
Xanthomonas arboricola pv. *pruni*	CITA 33	JHUQ01	5.10	65.4	4222	3720
Xanthomonas arboricola pv. *pruni*	IVIA 2626.1	LJGN01	5.02	65.4	4253	3849
Xanthomonas arboricola pv. *pruni*	CFBP 3894	LOMI01	5.05	65.4	4228	3967
Xanthomonas arboricola pv. *pruni*	MAFF301420	BAVC01	5.00	65.3	4315	3601
Xanthomonas arboricola pv. *pruni*	MAFF301427	BAVD01	4.90	65.4	5180	4686
Xanthomonas arboricola pv. *pruni*	MAFF311562	BAVB01	5.08	65.3	5422	4897

DISEASE CYCLE AND SURVIVAL OF THE EPIPHYTE *XAP*

Xanthomonas arboricola pv. *pruni* (*Xap*) is able to survive the winter in infected dormant buds, leaf scars, cankers and also on leaf debris that remains on the ground, all of these representing important sources of the survival and spread of the pathogen (Zaccardelli *et al.*, 1998). To date, the biological process associated with this capability of *Xap* has not been determined, but initial results from our research group permit the speculation that several factors, such as the formation of biofilm structures or the possible existence of viable but non-culturable bacterial cells, could play a role.

Bacteria from all of these sources of primary inoculum escape in the form of cryptoexudate and cause initial spring infections (Zaccardelli *et al.*, 1998). At this stage, bacteria multiply epiphytically on young leaves and penetrate through stomata or wounds, causing leaf lesions, which provide the secondary inoculum that produces the infection of fruits, twigs and trunks. Mummified almond fruits, which remain on the trees over time, can also serve as potential inoculum sources (Roselló *et al.*, 2012). Moreover, *Xap* is able to survive on leaf surfaces, forming organized bacterial aggregates or biofilms, or can penetrate inside woody tissues, which also constitutes secondary inoculum.

As in other xanthomonads, the disease cycle of *Xap* initiates when bacterial cells come into contact with the plant surface. At this time, several processes associated with pathogenesis are triggered, e.g. those related to the sensing of environmental conditions, bacterial adhesion to the surface and biofilm-like structure formation and chemotactic-mediated motility towards the inner space of the plant tissue. As the disease process advances, other virulence factors, such as cell wall-degrading enzymes and protein effectors that suppress plant immunity, may play a major role in the creation of appropriate environmental conditions for the establishment of a bacterial population. Despite the fact that studies of functional genomics have not been conducted on *Xap*, the information available on this matter from other xanthomonads models, such as *X. campestris*, *X. citri* and *X. oryzae* (Jacques *et al.*, 2016; Ryan *et al.*, 2011), in conjunction with the whole-genome analysis of *X. arboricola* (Cesbron *et al.*, 2015; Garita-Cambronero *et al.*, 2016a,b,c, 2017), has made possible the elucidation of the potential pathogenomics system of *Xap*, which is described in the section below.

PATHOGENOMICS OF *X. ARBORICOLA* ACCORDING TO THE WHOLE-GENOME COMPARATIVE ANALYSIS OF THE SPECIES

Environmental sensing is a crucial step at the beginning of the disease process of plant-pathogenic bacteria and, in general, TonB-dependent transporters (TBDTs), sensors of the two-component regulatory system (STCRs) and methyl-accepting proteins (MCPs) are amongst the key components during these initial stages. Homologues to TBDTs, related to carbohydrate scavenging and

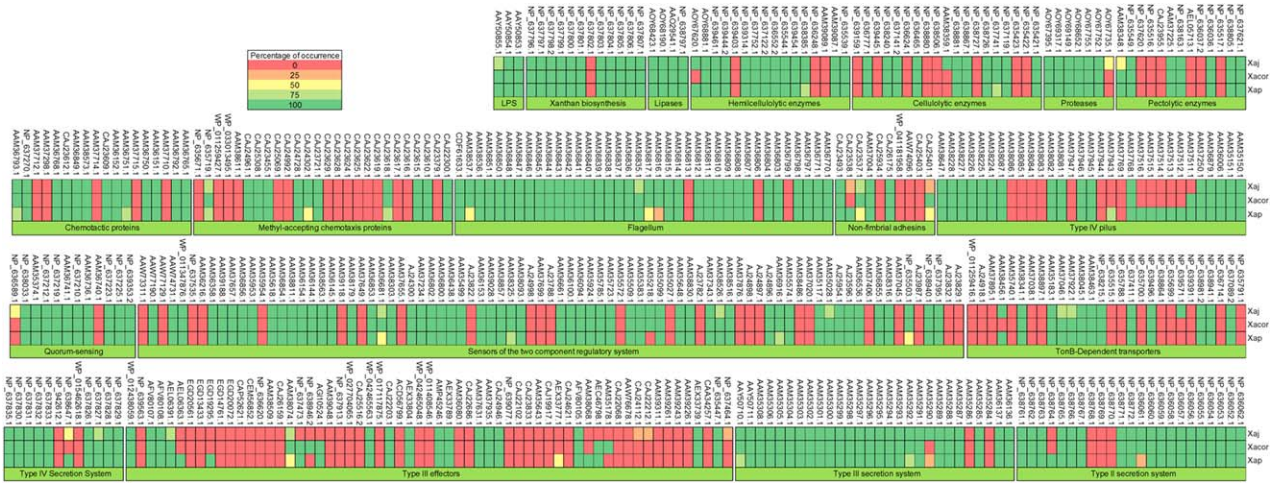

Fig. 3 Percentage of occurrence of the virulence-related genes described in *Xanthomonas* in the three most virulent pathovars of *Xanthomonas arboricola*. Each one of the genes is represented by its protein accession number at the National Center for Biotechnology Information (NCBI) database. Xacor, *X. arboricola* pv. *corylina*; Xaj, *X. arboricola* pv. *juglandis*; Xap, *X. arboricola* pv. *pruni*.

the transport of iron–siderophore complexes and vitamin B12 to the bacterial periplasm (Ryan *et al.*, 2011), have been found in the publicly available genomes of *X. arboricola*, as well as in two non-publicly available genomes of *X. arboricola* pv. *fragariae* (Vandroemme *et al.*, 2013a,b). On the whole, *X. arboricola* harbours at least 72 homologues to TBDTs. This large amount is comparable with those found in other *Xanthomonas* (Vandroemme *et al.*, 2013a,b). Pathovars *corylina*, *juglandis* and *pruni* present a similar content of TBDTs, with the exception of the homologue to gen XCC4237, which is only present in pathovar *juglandis*. In particular, in pathovar *pruni*, at least 13 specific TBDTs were identified, and most have been related to ferric and carbohydrate transportation (Garita-Cambronero *et al.*, 2016c) (Fig. 3). With regard to the STCRs, which are linkers between environmental sensing and other cellular processes, these were found in a large number in *X. arboricola* (Garita-Cambronero *et al.*, 2017). Pathovar *pruni* comprises at least 56 homologues to the STCRs described in *Xanthomonas* (Mhedbi-Hajri *et al.*, 2011). However, two STCRs, possibly associated with a light/oxygen response (AAM35218.1 and AAM36681.1) and present in the other more virulent pathovars *corylina* and *juglandis*, were not found in *Xap* (Fig. 3).

With respect to MCPs, only 11 homologues to those described in other *Xanthomonas* (Mhedbi-Hajri *et al.*, 2011) were found in *Xap*, as well as in the pathovars *corylina* and *juglandis* (Garita-Cambronero *et al.*, 2017) (Fig. 3).

Once the chemotactic signal is recalled by these chemoreceptors, it is transduced to the cytoplasmic chemotaxis transduction system (Scharf *et al.*, 2016), which, in *X. arboricola*, as in most of the xanthomonads, comprises the conserved proteins CheA, CheB, CheD, CheR, CheV, CheW, CheY and CheZ. This chemotactic system is also intrinsically linked to bacterial motility, controlling the direction of the flagellar motor rotation via the interaction of phosphorylated CheY with the flagellar switch system (Scharf *et al.*, 2016). With regard to the flagellum structure, all *X. arboricola* strains analysed harboured the 34 components associated with its regulation and structure (Fig. 3), but one exception to this has been observed in the non-motile strain CFBP 2528, which lacks a group of nine genes of the flagellar system (Cesbron *et al.*, 2015). Moreover, swimming and swarming studies conducted in the pathovars *juglandis* and *pruni* indicated that this bacterial organelle is functional in *X. arboricola* and could play an important role in the pathogenesis of the species (Cesbron *et al.*, 2015; Garita-Cambronero *et al.*, 2016c).

In addition to environmental sensing and bacterial motility, other factors, such as adhesion and the production of biofilm structures, may play central roles in the initial establishment of the bacterial population in the plant (Jacques *et al.*, 2016). Seventeen genes related to the bacterial type IV pilus, which is involved in bacterial attachment, movement across surfaces, orientation and multicellular organization, are present in all *X. arboricola* (Dunger *et al.*, 2016). *Xap* presents homologues to *pilB*, *pilE* and *pilI*, as well as protein sequences similar to the minor pilins *pilA*, *pilY1*, *pilX*, *pilW*, *pilV* and *fimT*, but with an identity percentage lower than 80% (Fig. 3). *Xanthomonas arboricola* pathovars *corylina*, *juglandis* and *pruni* share at least five homologues to non-fimbrial adhesins described in *X. campestris* pv. *vesicatoria* and *X. citri* ssp. *citri*. These non-fimbrial adhesins are involved in cell attachment, cell–cell interactions and aggregation (Berne *et al.*, 2015). In addition, pathovars *pruni* and *juglandis* share a homologue to the haemagglutinin protein CAJ23538.1 of *X. vesicatoria* (Fig. 3).

Xanthan gum, a virulence-related polysaccharide associated with bacterial attachment and biofilm formation, is produced in

most members of the *Xanthomonadaceae* (Moreira *et al.*, 2005). The *X. arboricola* strains analysed showed 11 of the 12 genes of the gum operon (Fig. 3). However, the apparent absence of *gumG* in this species may not affect the polymerization of this polysaccharide, as has been demonstrated in functional genomics studies performed in other *Xanthomonas* species (Katzen *et al.*, 1998). Furthermore, according to studies using microarray-based comparative genomic hybridizations, *gumG* may be present, at least, in strain 109 of *Xap* (Mayer *et al.*, 2011). Homologues to other virulence-related proteins (AAY50853.1, AAY50854.1, AAY50855.1) associated with extracellular polysaccharide production in *X. campestris* (Lu *et al.*, 2007) are also conserved in all the analysed strains of *X. arboricola* (Fig. 3).

In *Xanthomonas*, the coordinate expression of the mentioned virulence factors (exopolysaccharide production, motility and biofilm formation), as well as the secretion of extracellular enzymes and the entrance to the apoplast through the stomata, are regulated by the quorum sensing response (Büttner and Bonas, 2010). All *X. arboricola* strains studied, including *Xap*, conserve all the key genes in the synthesis, perception and transduction of the quorum sensing signal in *Xanthomonas*, although RpfC shows an identity lower than 80% in comparison with the other xanthomonads (He and Zhang, 2008) (Fig. 3).

Generally, once bacterial cells move to the inner tissues of the plant, the pathogen starts to secrete a battery of degrading enzymes and effectors that will change the physiology, metabolism and immune responses of the plant. *Xanthomonas* species release numerous cell wall-degrading enzymes through the xps type II secretion system (T2SS), as well as through outer membrane vesicles (Solé *et al.*, 2015). The analysed strains of *X. arboricola* presented all the genes associated with a functional xps T2SS, as well as those homologues related to the xcs T2SS, but the latter has not been associated with pathogenesis in *Xanthomonas* (Fig. 3). With regard to the degrading enzymes putatively translocated through this system, in *X. arboricola*, at least 13 cellulolytic enzymes, 13 hemicellulolytic enzymes, one hydrolase, four lipases, 10 pectolytic enzymes and seven proteases have been identified. Most pathogenic pathovars of the species shared 40 degrading enzymes, and differed only in the presence of a homologue of the rhamnogalacturonase B (AAM38348.1) in pathovars *corylina* and *pruni*, and by the presence of the cellulase AAM38359.1 solely in pathovar *juglandis* (Cesbron *et al.*, 2015; Garita-Cambronero *et al.*, 2017) (Fig. 3).

The most interesting differences amongst *X. arboricola* strains are related to the T3SS and its associated T3Es, as well as other type III secreted proteins (T3SPs). The *hrp* T3SS in *X. arboricola* is present in almost all the analysed strains, with the exception of some strains isolated from barley (Ignatov *et al.*, 2015), walnut (Cesbron *et al.*, 2015; Essakhi *et al.*, 2015), *P. mahaleb* (Garita-Cambronero *et al.*, 2016a) and peach (Garita-Cambronero *et al.*, 2017). All the latter strains showed a low-virulent or non-pathogenic phenotype after inoculation in the host in which they were isolated, and, in addition, presented a profile comprising zero to seven T3Es and T3SPs; furthermore, none were phylogenetically associated with the described pathovars of *X. arboricola* (Cesbron *et al.*, 2015; Garita-Cambronero *et al.*, 2017).

Comparative analysis amongst the pathovars *pruni*, *corylina* and *juglandis* indicates the presence of all the regulatory and structural components of the *hrp2* T3SS (Cesbron *et al.*, 2015; Garita-Cambronero *et al.*, 2016c; Hajri *et al.*, 2012). The T3E and T3SP profile in pathovar *pruni* comprises at least 19 genes homologous to those present in other xanthomonads. Meanwhile, pathovar *corylina* harbours 18 homologues and *juglandis* 17. Homologues to the T3Es *xopE3* (AAM36068.1) and *xopAQ* (EGD19295.1) were solely found in *Xap*; homologues to *xopAE* have been found only in pathovars *corylina* and *pruni*, whereas the T3Es *xopE2* and *xopC* are only present in pathovars *corylina* and *juglandis*, respectively. The remaining 16 T3Es and T3SPs are shared by all three pathovars. In addition, the presence of homologues to other T3Es has been described (for instance, *xopE2* in pathovar *pruni*), although showing a similarity or coverage percentage slightly below 80% (Fig. 3).

The presence of the VirB/VirD4 type IV secretion system has been identified in all the sequenced strains of *X. arboricola*, but the profile of genes varies amongst the subinfraspecific phylogenetic groups (Cesbron *et al.*, 2015; Garita-Cambronero *et al.*, 2017). With regard to the three pathovars with most phytopathogenic interest, none of the analysed strains harbour homologues to *virB5* and *virB7*, which code, respectively, for a minor subunit of the extracellular pilus and a short lipoprotein part of the pore that permits the secretion of the substrates to the extracellular space (Fronzes *et al.*, 2009). Pathovar *pruni* differs from pathovars *corylina* and *juglandis* in the presence of *virB6*, which has not been found in pathovar *corylina*, and is present only in strains Xaj417 and Xaj2 of pathovar *juglandis* (Fig. 3). In addition, Cesbron *et al.* (2015) described the presence of a variable profile of type IV effectors (T4Es) amongst pathogenic and non-pathogenic strains of *X. arboricola* isolated from walnut. These T4Es, present only in pathogenic strains of the pathovar *juglandis*, seem to be located in mobile genome elements probably obtained by horizontal gene transfer. To date, T4Es have not been found in the genome sequences of pathovars *corylina* and *pruni*. In respect of other secretion systems, such as the type VI secretion system (Gardiner *et al.*, 2014), none of the comparative analyses performed in *X. arboricola* have shown homologues to the components of this secretory system.

Most of the pathogenic processes are regulated by two-component transduction systems (TCSs). *Xanthomonas* is equipped with a large number of these components, which comprise up to 3% of the entire genome. Recently, Zheng *et al.*

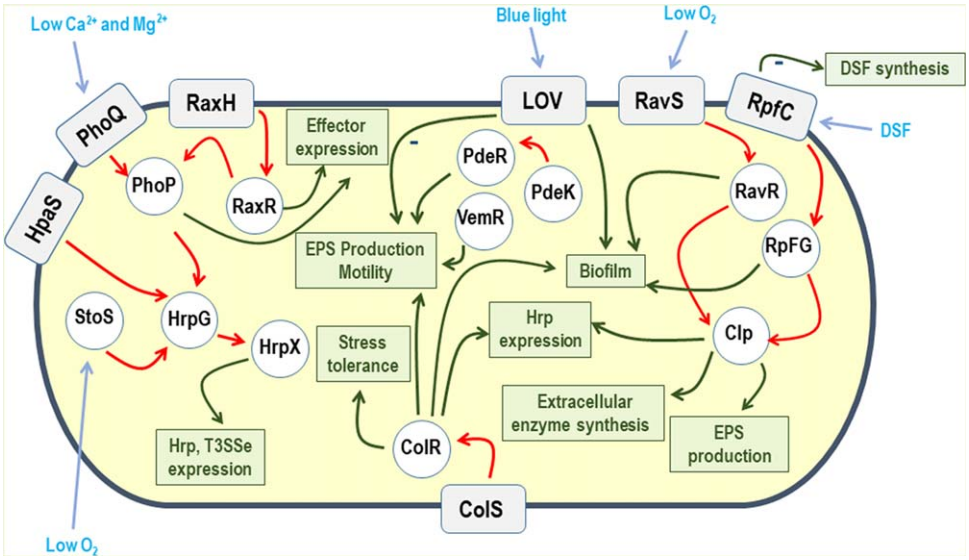

Fig. 4 Two-component transduction systems (TCSs) found in *Xanthomonas arboricola* according to the information obtained from the whole-genome sequence comparative analysis of 24 strains. Red arrows indicate protein interactions in each one of the TCSs; green arrows indicate the phenotypic result of the expression of each of the transduction pathways associated with pathogenesis in *Xanthomonas*; the minus sign '–' indicates the repression of a phenotypic character. This figure has been adapted to *X. arboricola* from the general transduction system present in the *Xanthomonas* genus according to Zheng *et al.* (2016). diffusible signal factor (DSF), extracellular polysaccharide; (EPS), type III secretion system effector; (T3SSe).

(2016) have presented an updated overview of the TCSs associated with chemotaxis, motility, carbohydrate metabolism, exopolysaccharide production, biofilm formation, the synthesis of extracellular enzymes and T3SS T3E expression. Figure 4 shows the TCS components described in *Xanthomonas*, which are present in *X. arboricola*, in order to show the potential regulatory pathways associated with virulence.

BACTERIAL SPOT OF STONE FRUITS AND ALMOND: DISEASE MANAGEMENT

The establishment and development of *Xap* outbreaks are strongly affected by agroclimatic conditions, cultivation practices and the variable susceptibility of *Prunus* species and cultivars. As for most bacterial diseases, control of bacterial spot of stone fruits and almond is very challenging because of the lack of effective chemical treatments and, once the bacterium is established in the orchard, it is very difficult to control. Therefore, the success of disease control is based on an integrated pest management approach intended to prevent the introduction and dissemination of the pathogen, which includes adequate legislation, the use of low-susceptible plants and suitable agronomic measures, including chemical or other treatments in the orchards, and forecast models.

Xanthomonas arboricola pv. *pruni* (*Xap*) is considered to be a quarantine regulated pathogen in some geographical areas, for instance by the European Union (EPPO, 2006; OJEC, 2000). Nursery production of propagating material requires regular inspection of *Prunus* species intended for planting, other than seeds (OJEC, 2000). As a result of the existence of latent infections that enable bacterial spread by propagating material (Dhanvantari, 1971; Goodman and Hatting, 1986; Zaccardelli *et al.*, 1998), rapid and highly sensitive methods for the detection of *Xap*, even in asymptomatic samples, are necessary (Palacio-Bielsa *et al.*, 2011).

The current EPPO standard protocol for the diagnosis of bacterial spot of stone fruits and almond (EPPO, 2006) is based on the isolation of the pathogen, followed by the identification of pure cultures using different techniques, such as biochemical tests, immunofluorescence (IF), protein profiling (sodium dodecylsulfate-polyacrylamide gel electrophoresis, SDS-PAGE), fatty acid methyl-ester profile analyses (FAME), rep-PCR and pathogenicity tests. However, the protocol does not include the conventional and real-time polymerase chain reaction (PCR) methods that are now available, which constitute a significant improvement. Comprehensive reviews of currently available information on the detection and identification of *Xap* have been published (Janse, 2010; López *et al.*, 2010; Palacio-Bielsa *et al.*, 2012).

More recently, new molecular and serological methods have been developed. Bühlmann *et al.* (2013) designed a rapid and sensitive protocol based on loop-mediated isothermal amplification (LAMP) enabling reliable discrimination between phylogenetically closely related *X. arboricola* pathovars and other bacteria associated with *Xap* host plants. Moreover, Garita-Cambronero *et al.* (2017) have designed a real-time PCR amplification protocol

based on the virulence-associated gene *xopE3*, described as specific for *Xap* (Pothier *et al.*, 2011), which can be used in conjunction with the method designed on the ABC transporter system (Palacio-Bielsa *et al.*, 2011) for the precise detection and identification of the xanthomonads detected in *Prunus* spp. With regard to serological techniques, a lateral flow immunoassay has been designed for the detection of *Xap* in symptomatic samples (López-Soriano *et al.*, 2017).

The use of tolerant or resistant cultivars in disease-threatened areas is strongly recommended. Nevertheless, most peach, apricot and Japanese plum genotypes are either susceptible or very susceptible to *Xap*. There is abundant literature on the susceptibility of different cultivated *Prunus*, but only limited information on the susceptibility of almond cultivars (Bazzi *et al.*, 1990; Garcin and Bresson, 2011; Palacio-Bielsa *et al.*, 2010; Ritchie, 1995; Simeone, 1990; Topp *et al.*, 1989). Few reports of the successful breeding for resistance to *Xap* are available. Moreover, the scarcity of stone fruit germplasm with high levels of disease resistance is a constraint for the preventative control of the bacterium. Mapping of quantitative trait loci (QTLs) associated with resistance against *Xap* in apricot and peach has been performed in order to develop assisted molecular markers that could be applied for breeding (Socquet-Juglard *et al.*, 2013; Yang *et al.*, 2013).

Cropping conditions, such as irrigation, fertilization and the timing and frequency of pruning, are factors that also play a role in outbreaks and the severity of the disease. Cultural practices must be addressed to reduce inoculum levels. Symptomatic plant material, including fallen leaves, as well as fruits that remain on the tree after harvest, should be removed. Particularly, in affected plum orchards, where cankers may develop extensively, branches with lesions caused by *Xap* must be pruned during the winter, whereas, in spring and summer, an accurate visual inspection is advised in order to detect newly formed bacterial cankers. Disinfection of pruning tools should be performed to prevent bacterial dissemination.

Amongst the factors that promote the occurrence of severe outbreaks of bacterial spot disease, sand or sandy loam soils with high clay content are considered to favour bacterial spot susceptibility of *Prunus* (Lamichhane, 2014). Overhead irrigation is a serious obstacle for disease control and must be avoided. Moreover, imbalance in nutrients (with an excess of nitrogen) renders host plants more susceptible to infections, and so moderate inputs of fertilizers during the growing seasons are preferable to a single, abundant application in spring (Stefani, 2010). Protection provided by windbreaks has also proved to be an effective measure against *Xap* dissemination inside orchards, as they reduce its spread by wind-blown rain (Ritchie, 1995, 1999).

The use of antibiotics for plant protection, such as streptomycin or oxytetracycline, has proven to be effective against *Xap* (Dhanvantari *et al.*, 1978), but is prohibited, amongst other countries, in the whole European Union. The strategy most widely used in the field is chemical control based on the application of copper compounds, which is preferential and intensively applied in autumn and spring before flowering. Copper could be applied during the growing season if a disease outbreak occurs in order to reduce secondary bacterial inoculum (Stefani, 2010). However, it should be considered that copper may cause phytotoxicity, particularly in peach and nectarine. Moreover, the presence of *Xap* isolates resistant to copper in Italian orchards has been confirmed recently (Giovanardi *et al.*, 2016a).

The use of zinc sulfate has also been reported for the preventative control of bacterial spot on peach in Uruguay (Palacio-Bielsa *et al.*, 2015). The use of sulfur compounds, which are registered for use against fungi and mites but not bacteria, is advisable in integrated pest management in those orchards showing fungal diseases, as they also have some efficacy in controlling *Xap* (Mclaren *et al.*, 2005).

Increasing limitation of chemical treatments has stimulated interest into the application of biocontrol agents against bacterial diseases. The use of bacterial antagonists against *Xap*, such as strains of *Pseudomonas fluorescens* (Biondi *et al.*, 2009), *Pseudomonas aeruginosa* (da Silva Vasconcellos *et al.*, 2014) and *Xanthomonas campestris* (Kawaguchi *et al.*, 2014), has been experimentally assayed. Selected bacteriophages have also been considered as candidates for the control of *Xap*. However, their large-scale use could be troublesome because of reduced epiphytic phage survival, interference with commonly used pesticides and the risk of bacterial immunity development (Zaccardelli *et al.*, 1992).

Although it is still at the developmental stage, another possible approach is the use of certain bioactive molecules, such as glucohumates, described as biostimulants or inducers of systemic resistance and foliar fertilizers (Giovanardi *et al.*, 2016b).

Moreover, the use of elicitor peptides (Peps) has been evaluated against *Xap* in *ex vivo* assays on *Prunus* spp. These peptides boost resistance on peach and almond plants, which show an enhancement of their resistance to the bacterial pathogen. Application of such peptides could be used when disease-predictive models indicate a high probability of pathogen infection (Ruiz *et al.*, 2017).

Finally, the development and implementation of predictive disease models might be of great help to correctly plan field treatments to control *Xap* in stone fruit and almond orchards, avoiding spraying when there is reduced or no risk of disease. The capacity of *Xap* for spread, weather conditions and disease outbreaks are closely associated, and so the disease does not show a very consistent pattern (Battilani *et al.*, 1999). A forecast model has been developed for peach in France (Garcin *et al.*, 2011) and studies for

the development of a prediction model have been performed in Spain (Morales *et al.*, 2017). All of these studies must be validated during several seasons for different *Prunus* species and under local conditions.

FUTURE PROSPECTS

Studies to improve our knowledge about *X. arboricola* species infection mechanisms have revealed an understanding of how these bacteria specifically interact with their precise hosts. The recent availability of genomic data on *Xap* strains, in addition to data for other closely related *Xanthomonas* spp. from the same host or bacterial species, has provided some clues to the key steps followed by these pathogenic bacteria in the different stages of infection establishment. Further functional studies are needed to conclusively confirm the essential specific elements involved in plant–bacteria interaction. This information will allow the future development of new control strategies based on the targeting of the mechanisms comprising the relationship of the bacteria with the host, for instance by blocking those that participate in adhesion, chemotaxis, biofilm formation or enzyme delivery to the plant cell or in the suppression of plant immunity.

Moreover, genomic studies have elucidated the existence of a diversity of *X. arboricola* strains associated with *Prunus* spp., which will permit the definition of those strains hazardous to plant health and epidemiologically relevant, and therefore needing to be regulated.

Genomic analysis of *X. arboricola* is a good example of the practical implications of such studies in plant disease control, not only for the future development of innovative control strategies, but because their use in the precise identification of pathogenic bacteria can avoid putative costly mistakes caused by the adoption of unnecessary control measures.

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