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CURRENT STATUS OF BACTERIAL SPOT OF STONE FRUITS AND ALMOND CAUSED BY *XANTHOMONAS ARBORICOLA* pv. *PRUNI* IN SPAIN

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SUMMARY

In 2002, typical symptoms of bacterial spot disease of stone fruits caused by *Xanthomomas arboricola* pv. *pruni* (*Xap*) were observed for the first time on Japanese plum in Badajoz (south-western Spain). During the following years, the pathogen was found in seven other eastern and northern Spanish provinces (Valencia, Alicante, Zaragoza, Huesca, Navarra, Lérida and Mallorca) affecting different cultivars of Japanese plum, nectarine, peach and almond. There are few previous reports of *Xap* on almond, the Spanish outbreaks constituting its first detection on this host in the European Union (EU). Identification of the pathogen was performed using biochemical tests, fatty acid methyl esters (FAME) profiles, conventional and real-time PCR, and hypersensitivity reaction on tobacco leaves. Pathogenicity was demonstrated by inoculation of young potted plants of peach, plum or almond and successful re-isolations from plants with symptoms. In areas where infected plants were found, eradication programs were set up since *Xap* has a quarantine status according to phytosanitary EU legislation.

Key words: symptomatology, biochemical tests, FAME, PCR, inoculation, diagnosis.

Xanthomonas arboricola pv. *pruni* (*Xap*), the agent of bacterial spot disease, is a quarantine organism in the EU phytosanitary legislation (Anonymous, 2000 and amendments) and in the European and Mediterranean Plant Protection Organization list [EPPO A2 List of pest recommended for regulation (Anonymous, 2003a)]. *Xap* can affect all cultivated *Prunus* species, peach, plum, almond, apricot and cherry in particular, and their hybrids. Other exotic or ornamental species, such as *P. davidiana* and *P. laurocerasus*, can also be affected (Anonymous, 2003a, 2006a, 2009). The most severe epidemics have been reported on the Sino-Japanese plum group (*P. salicina* and *P. japonica*) and their hybrids, peach (*P. persica* and hybrids) and nectarine (*P. persica* var. *nectarina*) (Ritchie, 1995; Stefani, 2010).The inconsistent pattern of bacterial epidemics observed in different countries or areas may be related to differential pathogenicity features of bacterial strains, variations in susceptibility of stone fruit species and cultivars, and cropping conditions, such as irrigation, fertilization and pruning time and frequency (Stefani, 2010).

The economic impact of bacterial spot largely depends on three major parameters: reduced quality and marketability of fruits, reduced orchard productivity, and increased costs of nursery productions (Stefani, 2010). There is not much information about the real costs of a disease outbreak regarding damages or economic crop losses. In the United States, Dunegan (1932) observed that 25-75% of the fruits could show lesions in neglected peach orchards. According to Stefani (2010), an epidemic in a commercial plum orchard of northern Italy affecting 30% of the fruits, could easily result in crop losses estimated over 11,200 € per ha (cv. Golden Plum) or 9,500 € per ha (cv. Angeleno).

Currently, the disease has been reported in most of the stone fruit-producing countries from Africa (South Africa and Zimbabwe); America (Bermuda, Canada, Mexico, USA, Argentina, Brazil and Uruguay); Asia [China, India, Japan, People's Democratic Republic of Korea (North Korea), Republic of Korea (South Korea), Lebanon, Pakistan, Saudi Arabia, Taiwan and Tajikistan]; Europe (Bulgaria, France, Italy, Moldova, Montenegro, The Netherlands, Romania, Russia, Slovenia, Spain, Switzerland and Ukraine) and Oceania (Australia and New Zealand) (Young, 1977; Jindal *et al.*, 1989; Akthar *et al.*, 1995; Panič *et al.*, 1998; Anonymous 2006a, 2006b, 2009; Roselló, 2007; Roselló *et al.*, 2007, 2010; Palacio-Bielsa *et al.*, 2010a, 2010b; Pothier *et al.*, 2010). Regarding the identification of *Xap* in almond, the old records from Japan and Sicily (insular Italy) were not

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Fig. 1. Cronological detections of outbreaks of *Xanthomonas arboricola* pv. *pruni* in Spain. 1, Badajoz; 2, Valencia; 3, Alicante; 4, Zaragoza; 5, Huesca; 6, Lérida; 7, Navarra; 8, Mallorca.

substantiated when the original publications were re-examined. As to Japan, *Xap* was actually identified in plum rather than almond (Ishiyama, 1923), whereas the agents of the different almond diseases found in Sicily did not comprise *Xap* (Ciccarone, 1958, 1959).

Spain is the second world producer of almond (first in the EU), the fourth world producer of peach and nectarine (second in the EU), the fifth world producer of cherry (second in the EU), the eighth world producer of plum (third in the EU) and the thirteenth world producer of apricots (third in the EU) (FAOSTAT, 2009). The total cultivated area in regular plantations in 2009 was of 562,616 ha for almond; 76,730 ha for peach and nectarine; 24,304 ha for cherry; 19,226 ha for apricot and 18,489 ha for plum (MARM, 2010).

In 1920, the bacterial spot disease of stone fruits was reported for the first time in Europe in Italy (Battilani *et al*., 1999) but only in 2002 *Xap* was detected in Spain for the first time, in Japanese plum (*P. salicina*) orchards in the province of Badajoz (Roselló *et al*., 2007). Typical disease symptoms were observed on fruits and leaves of cvs Larry Ann and Friar, and an eradication program was enforced, according to the EU legislation. Since then, surveys conducted in this area detected the disease sporadically in some orchards in 2003 and 2007.

In the following years, new outbreaks were found in other Spanish regions. In 2004, symptoms were observed on the leaves of young potted plants of nectarine (cvs Zephir and Newtop) and Japanese plum (cv. Anna Gold) in a nursery in Valencia. In 2006, *Xap* was detected in almond orchards in Alicante and, in 2008, in peach, plum and almond in Zaragoza and Huesca, as well as in peach and nectarine in Lérida. In 2009, new outbreaks were recorded in peach and almond in Navarra, and again, in Lérida, Zaragoza and Huesca. In 2010, *Xap* was detected on plum in Mallorca (Balearic Islands) and again, on almond in Zaragoza. Spanish outbreaks on almond constituted the first report of the pathogen on this host in the EU (Palacio-Bielsa *et al*., 2010a, 2010b). Fig. 1 shows the geographical locations of the outbreaks detected in Spain until now. In all cases, *Xap* was isolated and identified and eradication programs were implemented.

The symptoms observed in plum, peach and nectarine corresponded to those typical of bacterial spot disease of stone fruits. However, the symptoms shown by almonds, although having some features in common with those reported for other stone fruit species are quite different, especially in the fruits (Anonymous, 2004; Edstrom, 2007; Palacio-Bielsa *et al.*, 2010a, 2010b; Roselló *et al.*, 2010). As these differences could lead to misdiagnosis and there is not much information on the symptoms shown by almond, we now detail them in comparison with those displayed by other hosts (Fig. 2 to 4).

Xap symptoms on almond are first noted in spring and can be observed until leaf fall, as in other stone fruits. Symptoms on leaves and twigs are similar to

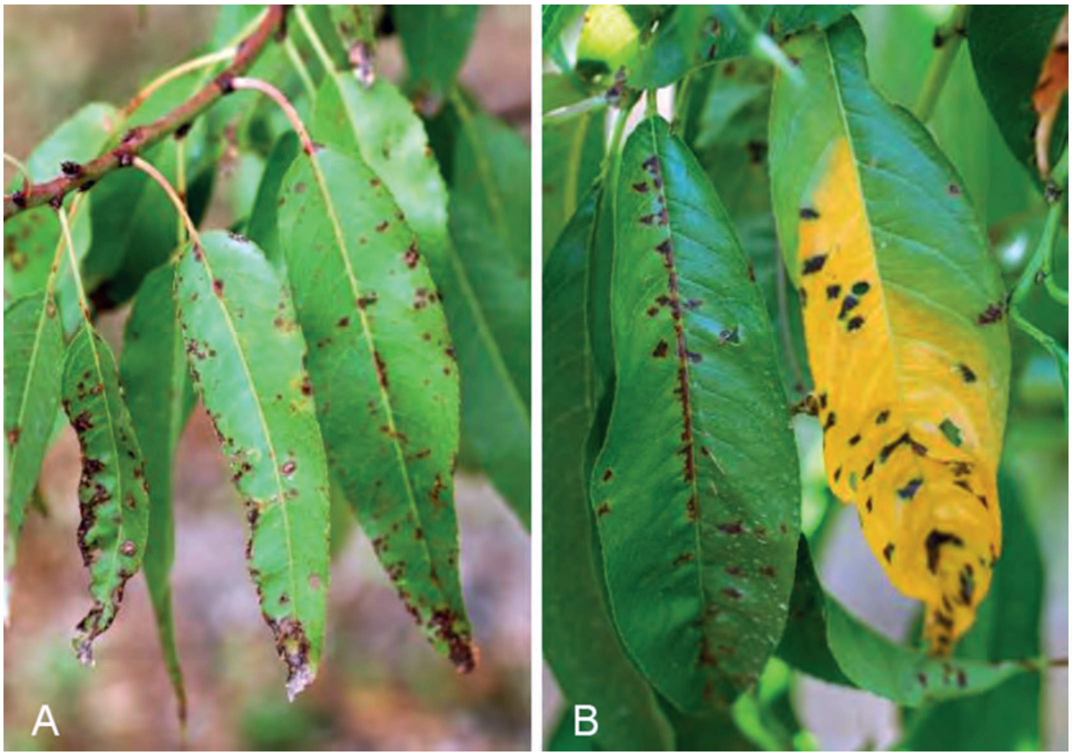

Fig. 2. A. Typical almond leaf spots clustering. B. Typical yellowish symptoms on peach leaf.

those observed on other hosts. Lesions are visible on both sides of the leaves and appear generally clustered in areas that remained wet for longer periods (Fig. 2A). The majority of affected leaves are located on the basal part of the twigs and on more than one-year-old wood. Contrary to the typical yellowing shown by peach (Fig. 2B), this is not observed on almond leaves, as well as the premature leaf drop, although it can occur in severely affected trees. When foliar lesions coalesce, large necrotic areas are formed resulting in "shot-holes" upon detachment of dried tissues. These bacteria-induced symptoms can easily be confused with those of fungal diseases incited by *Wilsomomyces carpophilus* or *Venturia carpophila* that also cause a "shot-hole" condition.

Twig lesions are not observed on almond, as frequently as leaf and fruit symptoms. When lesions appear on current season's wood, 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, inciting dieback. Cankers can sometimes be observed in the branches, as in other hosts.

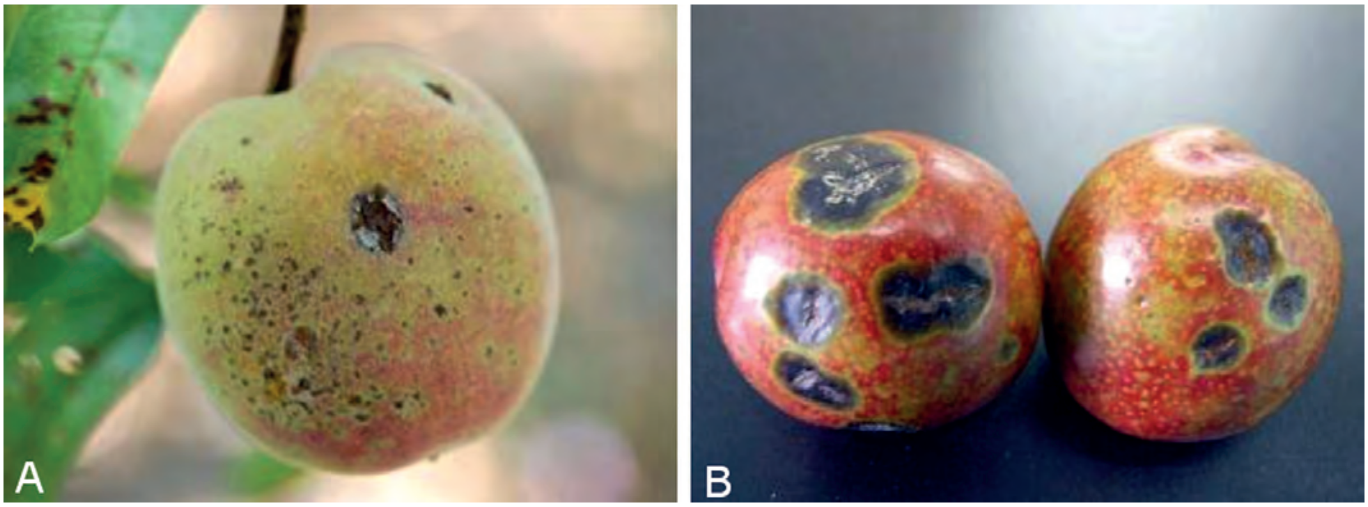

Fig. 3. A. Lesions on peach fruits. B. Lesions on Japanese plum fruits.

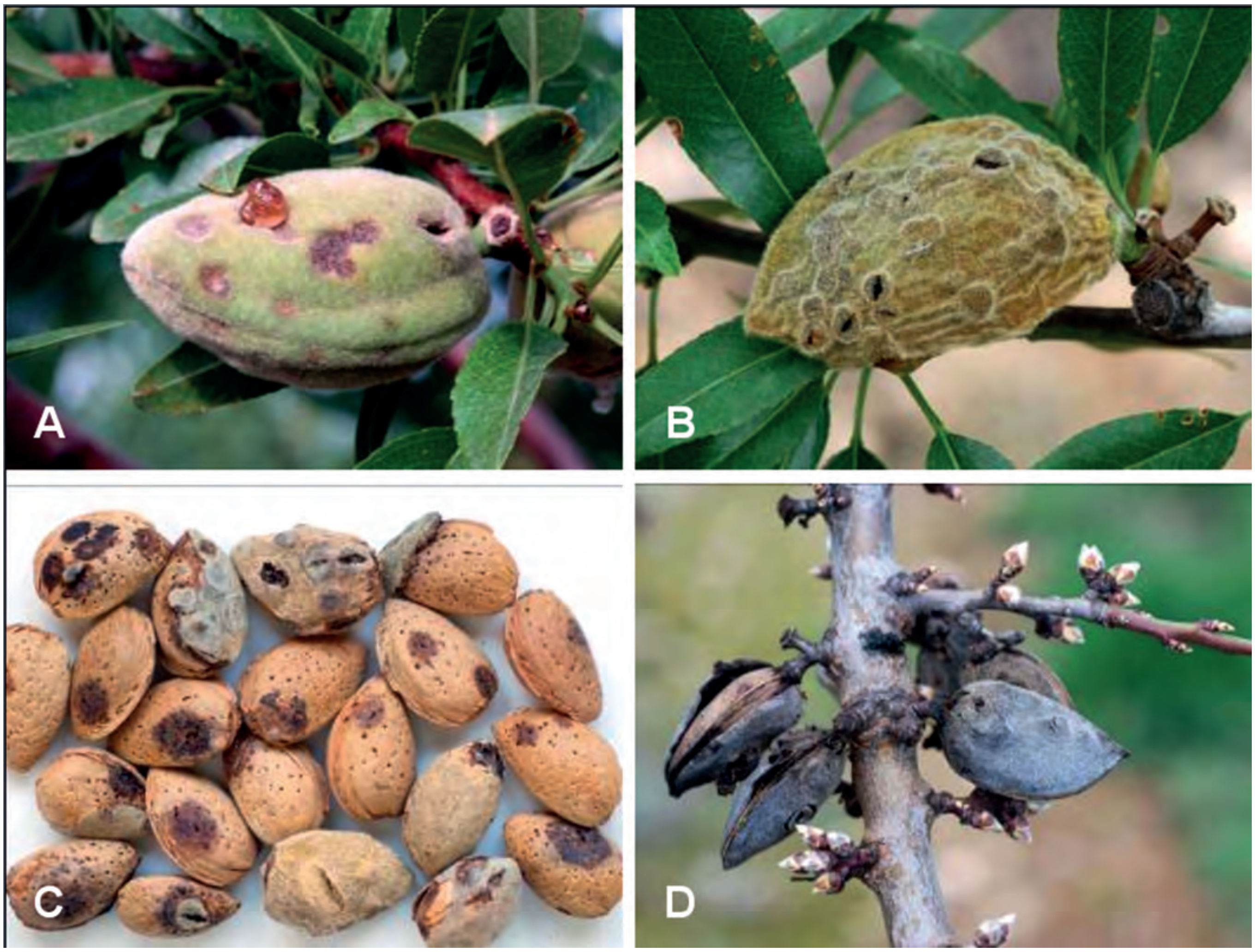

Fig. 4. A. Initial sunken and corky almond nut lesions oozing gum. B. Raised nut lesions on dehydrated mesocarp. C. Circular dark spots on the endocarp of almond nuts. D. Infected nuts remaining on trees after harvest (mummies).

Symptoms on almond fruits are quite specific and different from those observed on other stone fruits, such as peach (Fig. 3A) or plum (Fig. 3B). During spring, infected fruits initially display sunken, corky lesions, oozing gum that streams or clumps (Fig. 4A). In summer, when the mesocarp dehydrates, the sunken lesions become raised (Fig. 4B). In some cases, circular dark spots are observed on the endocarp, which can even affect the nut (Fig. 4C). Infected fruits either drop prematurely or remain on the trees after harvest (Fig. 4D). These mummies harbour viable bacteria, therefore serving as inoculum sources thereafter.

Isolations performed from fruit, canker and leaf samples collected from diseased plum, nectarine, peach or almond, consistently yielded *Xanthomonas*-like colonies on yeast extract peptone glucose agar (YPGA) (Ridé, 1969; Lelliot and Stead, 1987) and King's medium B (King *et al*., 1954) supplemented with sterile 250 mg l^{-1} cycloheximide (Sigma-Aldrich, USA), after incubation at 25ºC for 72 h. Identification of purified colonies was performed by phenotypic, molecular and pathogenicity tests.

With a selection of 101 isolates and a reference Italian strain, originally isolated from *P. salicina* (IsPaVe B4), 14 conventional biochemical tests were done according to Schaad *et al*. (2001). Moreover, API 20 NE miniaturized strips (bioMérieux, France) were utilized according to the manufacturer's instructions except that incubation was at 25ºC for 48 h instead of 29ºC for 24-48 h. API 50 CH miniaturized strips (bioMérieux, France) were used with Dye's C medium (Dye, 1968) with 0.08 ‰ bromothymol blue, and incubation at 25ºC for 72 h. In most cases, results were the same for all the isolates and agreed with those reported for *Xap* (Bradbury, 1986; Van den Mooter and Swings, 1990; Vauterin *et al*., 1995). Table 1 shows the phenotypic characteristics determined for all the analyzed isolates. Conventional and real-time PCR, performed on the same isolates following a protocol by Pagani (2004) modified by López *et al.* (this issue) and a new real-time PCR proto-

Table 1. Phenotypic characteristics of 101 *X. arboricola* pv. *pruni* Spanish isolates from *Prunus* spp.

Test	Results
Gram (KOH)	Gram negative
Oxidase	-
Catalase	+
O/F from glucose (Hugh-Leifson)	+/- (5 days) (weak oxidative)
Nitrate reduction	- (5 days)
Arginine dihydrolase (Thornley)	- (5 days)
Urease	- (5 days)
Indol	- (5 days)
Levan	+ (3 days)
Gelatin hydrolysis	+ (3 days)
Tween 80 hydrolysis	+ (3 days)
Esculin hydrolysis	+ (24 h)
Simmons' citrate	+ (5 days)
Growth in Nutrient broth at 37ºC	- (5 days)
API 20 NE* (bioMérieux, France)	**+**: Esculin hydrolysis, Gelatine hydrolysis, β-Galactosidase activity. Utilization under aerobic conditions of: Glucose, Mannose, N-Acetylglucosamine, Malate and Citrate.
API 50 CH** (bioMérieux, France)	**+**: Esculin hydrolysis and N-Acetylglucosamine. §V: Acidification of: Galactose, D-Glucose, D-Fructose, D-Mannose, Cellobiose, Sucrose, Trehalose, D-Fucose and L-Fucose. Alkalinization of 2-keto-gluconate.

+, positive reaction in 100% of isolates; -, negative reaction in 100% of isolates; *readings after 48 h of incubation at 25ºC; **readings after 72 h of incubation at 25°C; §V, positive reaction in more than 85% of isolates.

col developed by Palacio-Bielsa *et al.* (2011), yielded the expected amplicons of 943 and 70 bp, respectively, from all isolates. Fatty acid methyl ester (FAME) profiles of a representative selection of 9 *Xap* isolates from different hosts incubated at 28ºC for 48 h, identified the isolates as *Xap* (similarity index between 0.678 and 0.824) when the profiles were compared by means of the MIS Identification database TSBA.40 (vers. 4.10). These results agree with those of Scortichini *et al.* (1996), who reported that the branched fatty acids 15:0 ISO and 15:0 ANTEISO and the unsaturated fatty acid 16:1 w7c, were the most in the studied isolates. Although Stead *et al.* (1992) reported misidentification when FAME profiles were compared by means of the MIS database TSBA.40, since this database uses data from isolates incubated for 24 h, this did not occur in our case, because all the tested strains were correctly identified.

A typical (or sometimes atypical) hypersensitivity response was obtained after 1 to 4 days in tobacco leaves (cv. Xanthi) with suspensions of *Xap* isolates (*ca.* 10^9 CFU ml^{-1}) inoculated according to Klement *et al.* (1964). Pathogenicity was confirmed by inoculation of suspensions of the isolates (*ca.* 10^7 CFU ml^{-1}) into leaves of young potted plants and in detached leaves of plum, nectarine or almond (depending on the origin of the isolates) following Anonymous (2006a) and Randhawa and Civerolo (1985) procedures. The pathogen was re-isolated from leaf tissues showing symptoms that developed after one week of incubation at 25ºC and 16:8 h photoperiod. Re-isolated colonies showed the same morphology and characteristics as those of the inoculated strains.

The results of all these tests permitted to confirm the presence of *Xap* in the outbreaks of bacterial spot disease on stone fruit and almond detected in Spain. Eradication measures were implemented according to the EU, Spanish and local legislations (Anonymous, 1998, 2000, 2002, 2003b, 2005, 2007, 2008, 2010) following which, some areas can now be considered pathogen-free since new outbreaks have not been detected for some years. Other areas, however, are still the site of active programs to prevent disease spread.

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