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EVALUATION OF THE EFFICIENCY OF A CONVENTIONAL PCR PROTOCOL FOR THE DIAGNOSIS OF BACTERIAL SPOT DISEASE CAUSED BY *XANTHOMONAS ARBORICOLA* pv. *PRUNI* IN STONE FRUITS AND ALMOND

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SUMMARY

Xanthomonas arboricola pv. *pruni* (*Xap*), the causal agent of bacterial spot disease of stone fruits and almond, has a quarantine status for the European Union and the European and Mediterranean Plant Protection Organization. The symptoms in the diverse hosts show some differences and, although being quite typical, could be confused with those of some fungal diseases or other biotic or abiotic causes. Consequently, an accurate molecular diagnosis method is required for a rapid identification of the pathogen in samples of imported plants, from nurseries, orchards, etc. A protocol for conventional PCR designed by Pagani (2004) has been the only molecular analytic tool available for several years. It has been optimised for improving its specificity and sensitivity, and the results of its evaluation in 316 bacterial spot-like symptomatic samples of almond, apricot, cherry, Japanese plum and peach, compared with those of isolation and real-time PCR, are reported. The optimised PCR protocol showed specificity for a collection of *Xap* strains tested. Few non-desired reactions were obtained with some other xanthomonads which have not been reported from *Prunus* species. Sensitivity thresholds ranged from 10^2 to 10^5 CFU ml^{-1}, depending on the hosts and type of plant material. This conventional PCR assay proved to be an excellent candidate for a rapid screening and presumptive diagnosis in cases where real-time PCR equipment is not available.

Key words: symptomatology, PCR, isolation, specificity, sensitivity.

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Xanthomonas arboricola pv. *pruni* (*Xap*), a quarantine organism in the European Union (EU) (Anonymous, 2000 and amendments) and the European and Mediterranean Plant Protection Organization (EPPO), is the causal agent of bacterial spot disease of stone fruits (Dunegan, 1932; Anonymous, 2003) and almond (Roselló *et al.*, 2010; see Palacio-Bielsa *et al.* and Roselló *et al.*, this issue), as well as of some ornamental species of *Prunus* (see also Palacio-Bielsa *et al.* and Roselló *et al.*, this issue). The disease is currently widespread in several European countries, such as in Italy, where it was first reported in 1920 (Battilani *et al.*, 1999), and several outbreaks have occurred in the last decades in France, the Netherlands, Switzerland and Spain. The bacterium has also been identified in Bulgaria, Montenegro, Republic of Moldova, Romania, Russian Federation, Slovenia and Ukraine (see also Palacio-Bielsa *et al.* and Roselló *et al.*, this issue).

A laboratory diagnosis is required to confirm the presence of *Xap* because symptoms of bacterial spot disease can sometimes be confused with injuries caused by other bacteria, fungi, viruses or abiotic factors. Without an accurate identification of the causal agent, an incorrect diagnosis may be made and inappropriate management decisions taken (Anonymous, 2004, 2005, 2006; Palacio-Bielsa *et al.*, 2009, 2010a; Zúñiga and Lezaun, 2010). Different *Pseudomonas* species, such as *P. syringae* pv. *mors-prunorum*, *P. syringae* pv. *persicae*, *P. syringae* pv. *syringae* and *P. viridiflava* can also cause symptoms on stone fruits. These bacteria can produce dieback, cankers and, in certain cases, *P. syringae* pv. *mors-prunorum* and *P. syringae* pv. *persicae* can also cause leaf spots that may be confused with those due to *Xap* (Anonymous, 2005). *P. amygdali* causes a hyperplastic bacterial canker on almond (Psallidas, 1997).

Leaf spot symptoms similar to bacterial spot can also be produced by the fungi *Wilsonomyces carpophilus* and *Blumeriella hiemalis* (Jones, 1995; Ogawa and English, 1995). Other fungal diseases such as red leaf blotch of almond leaves (*Polystigma ochraceaum*) can mask *Xap* symptoms (Palacio-Bielsa *et al.*, 2010a). On cherry and

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peach, *Prunus necrotic ringspot virus* (PNRSV) causes chlorotic spots on the leaves which can evolve to "shot-hole" (Anonymous, 1996). Abiotic factors such as hail, water stress, nitrogen deficiency, spray injury from chemicals, or copper phytotoxicity can cause leaf spot symptoms resembling those of bacterial spot (Anonymous, 2005; Lalancette and McFarland, 2007). Peach cankers, caused by *Phomopsis amygdali* (Anonymous, 2005), and fruit symptoms on peach, caused by *Venturia carpophila*, can also be confused with those of bacterial spot (Anonymous, 2005; Shane and Sundin, 2011).

The only available European diagnostic protocol for *Xap,* published by the EPPO, does not include any molecular method for its detection (Anonymous, 2006). The protocol recommends the isolation of the bacterium, followed by identification by biochemical tests, protein and fatty acids methyl ester (FAME) profiling, repetitive extragenic palindromic PCR (REP-PCR), serological techniques and pathogenicity tests. A conventional PCR protocol for *Xap* detection and/or identification was developed at the North Carolina State University (USA) (Pagani, 2004). It uses primers based on the sequence of a *Xap* gene of a putative protein related to an ABC transporter ATP-binding system. Although there are now several new PCR protocols published in the recent years, i.e. PCR (Park *et al.*, 2010), real-time SYBR Green I Bio-PCR (Ballard *et al*., 2011), real-time TaqMan PCR (Palacio-Bielsa *et al*., 2011), duplex-PCR (Pothier *et al*., 2011a), and multiplex-PCR (Pothier *et al.*, 2011b), the Pagani (2004) protocol was previously modified (see below for details) and optimised in several EU laboratories, proving its usefulness for diagnosis of plants with *Xap* symptoms. Insufficient information, however, is available on the specificity, sensitivity, efficiency and robustness of this protocol when used for diagnosis of *Xap* on the tissues of its different hosts. Thus, the goal of this work was to: (i) analyse the specificity of the primers designed by Pagani (2004) for a large number of *Xap* strains from the EU and for other bacterial species; (ii) evaluate the sensitivity of the modified protocol in samples of different hosts; and (iii) compare the results of the analyses of field samples from different organs of five hosts by isolation, Pagani´s modified conventional PCR and the real-time PCR developed by Palacio-Bielsa *et al.* (2011). This information is required because this PCR methodology is currently widely used in many EU countries, and in the framework of COST 873. In Spain, it has been utilised for diagnosis of samples suspected to be infected by *Xap* before eradication in nurseries or orchards, for analysis of imported plants or nursery material, and in other countries for the accurate determination of the aetiology of suspected symptoms before setting up an integrated control of the disease.

The modified protocol, which utilises the primers and protocol described by Pagani (2004) with the below indicated modifications of the PCR reaction mixture and cycling conditions, was previously compared with the original one. The sequences of the primers were: forward Y17CoF 5'-GAC GTG GTG ATC AGC GAG TCA TTC-3´ and reverse Y17CoR 5'-GAC GTG GTG ATG ATG ATC TGC-3'. The expected amplicon size for *Xap* was 943 bp. The PCR reaction mix was performed in a final reaction volume of 50 µl including: 5 µl DNA extract; 1x PCR buffer (Biotools, Spain); 1.5 mM $MgCl_2$; 0.1 mM dNTP mix; 0.1 µM each primer; 1 U DNA polymerase from *Thermus* sp. (Biotools, Spain) for pure bacterial cultures or 2.5 U for plant samples. The amplification programme utilised was: an initial denaturation step of 4 min at 94°C, followed by 45 cycles of 1 min at 93°C, 1 min at 55°C and 2 min at 72°C, and a final extension step of 10 min at 72°C. Gel electrophoresis and ethidium bromide staining were performed as indicated by Pagani (2004). The programme was optimised for use with a Perkin Elmer 9600 model thermal cycler. A sample was considered positive when the expected band of 943 bp was observed in the gels after the amplification of the DNA and/or its tenfold dilution. A sample was considered neg-

Table 1. Detection of *Xanthomonas arboricola* pv. *pruni* strain IVIA 463 in a serial dilution (10 to 10^6 CFU ml^{-1}) in sterile distilled water or PBS and in leaf extracts from almond, apricot, Japanese plum, peach and peach x almond hybrid GF-677, using the optimised conventional PCR protocol.

Serial dilution of strain IVIA 463 in:	CFU ml^{-1}						Negative control[a]
	10	10^2	10^3	10^4	10^5	10^6	
Sterile distilled water or PBS	-	+	+	+	+	+	-
Spiked leaf extract from almond	-	w+	+	+	+	+	-
Spiked leaf extract from apricot	-	-	-	-	+	+	-
Spiked leaf extract from Japanese plum	-	-	-	-	w+	+	-
Spiked leaf extract from peach	-	+	+	+	+	+	-
Spiked leaf extract from GF-677	-	-	-	-	w+	+	-

[a]Negative control (sterile distilled water or PBS, or extract from healthy plant material); w+, weak band

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Table 2. Analysis of *Xanthomonas arboricola* pv. *pruni* in almond suspected samples by isolation, optimised conventional PCR and real-time PCR.

Almond cultivar	Organ	Samples (No.)	Orchards or origins (No.)	Isolation	Conventional PCR	Conventional PCR 1:10	Real-time PCR
nd	Leaf	5	2	+	-	-	+
nd	Leaf	1	1	+	-	+	+
nd	Leaf	1	1	+	-	-	-
nd	Leaf	12	2	-	-	-	-
nd	Leaf	14	5	-	-	nd	-
nd	Stem	3	1	-	-	-	-
nd	Canker	4	1	-	-	-	-
nd	Fruit	1	1	-	-	-	-
Ferraduel	Leaf	10	1	-	-	nd	-
Tuono	Leaf	5	1	-	-	-	-
Tuono	Stem	1	1	-	-	-	-
Tuono	Canker	7	1	-	-	-	-
Tuono	Fruit	4	1	-	-	-	-
Marcona	Leaf	7	1	-	-	-	-
Marcona	Fruit	3	1	-	-	-	-
Guara	Leaf	12	1	+	+	+	+
Guara	Leaf	12	1	-	-	-	-
Guara	Fruit	25	1	+	+	+	+

+, positive; -, negative; nd, not determined

ative if it did not yield the expected amplicon, provided that the appropriate assay controls tested positive.

Specificity tests were performed with 101 *Xap* strains from Italy [Istituto Sperimentale per la Patologia Vegetale (ISPaVe), Rome, and Istituto di Science per le Produzioni Alimentari CNR, Bari (ISPA)] and Spain [IVIA Collection (Instituto Valenciano de Investigaciones Agrarias, Valencia)]. Moreover, 32 isolates from stone fruit samples and the following 12 strains from other plant pathogenic species were also tested: *Agrobacterium tumefaciens* (C58), *A. vitis* (IVIA 339-26), *Brenneria quercina* (IVIA 2389-1a), *Clavibacter michiganensis* subsp. *michiganensis* (IVIA 2869), *C. michiganensis* subsp. *sepedonicus* (IVIA 2140), *Pseudomonas corrugata* (IVIA 1765), *P. savastanoi* pv. *savastanoi* (ITM 317), *P. syringae* pv. *mori* (IVIA 2488-1), *P. syringae* pv. *syringae* (IVIA 2716), *P. syringae* pv. *tomato* (IVIA 1733-3), *Xanthomonas arboricola* pv. *juglandis* (IVIA 1317-1a), *X. axonopodis* pv. *citri* (IVIA 2911), and two strains of saprophytic bacteria (*Pantoea agglomerans* strain IVIA FSO55 and *Pseudomonas fluorescens* strain IVIA 2521-1a). A suspension of each strain determined by spectrophotometry and dilution plating (*ca.* 10^8 CFU ml^{-1}) was prepared for PCR in sterile distilled water or phosphate buffered saline (PBS).

To evaluate the PCR sensitivity, plant extracts from tissue of almond cv. Marcona, apricot cv. Rojo de Carlet, Japanese plum cv. Red Beaut, peach cv. O´Henry and the peach x almond hybrid GF-677, were prepared by comminuting 1 g of leaf tissue from healthy plants in 5 ml of sterile distilled water or PBS. The plant extracts were additioned with known amounts of *Xap* strain IVIA 463 grown for 72 h at 25°C on yeast-peptone-glucose agar (YPGA) medium (Ridé, 1969; Lelliot and Stead, 1987), from bacterial suspensions at final concentrations ranging from 10 to 10^6 CFU ml^{-1}, as determined by dilution plating. Sterile distilled water or PBS bacterial suspensions of the same *Xap* strain with concentrations ranging from 10 to 10^6 CFU ml^{-1} were prepared as controls. DNA was extracted according to Llop *et al.*

Table 3. Comparative analyses of almond field samples and percentage of congruence isolation-optimised conventional PCR, isolation-real-time PCR and real-time PCR-optimised conventional PCR.

		Conventional PCR	
		+	-
Isolation	+	38	6
	-	0	83

+, positive; -, negative
Congruence isolation-optimised conventional PCR: 95.28%

		Real-time PCR	
		+	-
Isolation	+	43	1
	-	0	83

+, positive; -, negative
Congruence isolation-real-time PCR: 99.21%

		Conventional PCR	
		+	-
Real-time PCR	+	38	5
	-	0	84

+, positive; -, negative
Congruence real time-PCR-optimised conventional PCR: 96.06%

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Table 4. Analysis of *Xanthomonas arboricola* pv. *pruni* in apricot suspected samples by isolation, optimised conventional PCR and real-time PCR.

Apricot cultivar	Organ	Samples (No.)	Orchards or origins (No.)	Isolation	Conventional PCR	Conventional PCR 1:10	Real-time PCR
nd	Leaf	2	1	+	-	-	-
nd	Leaf	2	1	+	-	-	+
nd	Leaf	2	1	-	-	-	-
nd	Canker	3	1	-	-	-	-
nd	Fruit	7	1	+	+	+	+
nd	Fruit	1	1	+	-	+	+
nd	Fruit	3	1	-	-	-	-
Robada	Stem	1	1	-	-	nd	-
Robada	Bud	5	1	-	-	nd	-
Robada	Canker	4	1	-	-	nd	-

+, positive; -, negative; nd, not determined

(1999) as reported by Palacio-Bielsa *et al.* (this issue). Amplification runs were as outlined above.

The efficiency of the optimised protocol was then evaluated in comparison to isolation and real-time PCR according to Palacio-Bielsa *et al.* (2011) in a LightCycler 480 (Roche), in 316 samples of several cultivars of almond, apricot, cherry, Japanese plum and peach. Tables 2, 4, 6, 8 and 10 list the samples of each host from several Spanish regions, analysed between 2004 and 2011. All samples were collected in orchards or nurseries and were received as suspected of bacterial spot disease. Isolations were performed by plating the comminuted samples and their 1:10 and 1:100 dilutions on YPGA medium (Ridé, 1969; Lelliot and Stead, 1987), according to Palacio-Bielsa *et al.* (this issue). DNA extraction of the plant samples was performed as indicated above to be used by conventional PCR (modified protocol) and real-time PCR according to Palacio-Bielsa *et al.* (2011). For diluting the abundant inhibitors usually found in stone fruits organs, the conventional PCR was also performed on 1:10 diluted DNA extracts. A sample was considered positive if the threshold cycle (C_T), at which fluorescence was first detected, was achieved before the cycle 40.

For all PCRs assays, positive controls were heat-treated (96ºC for 10 min and subsequent cooling on ice) suspensions of *Xap* strain ISPaVe B4 or DNA extracted (Llop *et al.*, 1999) from spiked samples of the same hosts analysed, and negative controls were other heat-treated bacterial species and/or DNA extracts from healthy plant material of the same hosts analysed.

Percentages of congruence between results obtained by isolation and conventional PCR, between isolation and real-time PCR and between real-time PCR and conventional PCR were calculated for the samples of the different hosts.

Table 5. Comparative analyses of apricot field samples and percentage of congruence isolation-optimised conventional PCR, isolation-real-time PCR and real-time PCR-optimised conventional PCR.

		Conventional PCR	
		+	-
Isolation	+	8	4
	-	0	18

+, positive; -, negative

Congruence isolation-optimised conventional PCR: 86.67%

		Real-time PCR	
		+	-
Isolation	+	10	2
	-	0	18

+, positive; -, negative

Congruence isolation-real-time PCR: 93.33%

		Conventional PCR	
		+	-
Real-time PCR	+	8	2
	-	0	20

+, positive; -, negative

Congruence real time-PCR-optimised conventional PCR: 93.33%

The modified Pagani protocol showed specificity for all 101 Italian and Spanish *Xap* strains tested. Although Ballard *et al.* (2011) stated that eight non European strains (from Australia, Brazil, Canada and the United States) were not amplified with the primers designed by Pagani (2004), we have not found any negative result with the European strains tested. However, in the first assays, using the protocol without modifications, PCR products of around 943 bp were also amplified from non *Xap* bacterial strains as *P. savastanoi* pv. *savastanoi* ITM 317, *P. syringae* pv. *mori* IVIA 2488-1, *P. syringae* pv. *tomato* IVIA 1733-3 and *X. citri* subsp. *citri* IVIA 2911, and other PCR products of different sizes were also amplified from *X. arboricola* pv. *juglandis* IVIA 1317-1a and from the saprophytic *P. agglomerans* IVIA FSO55. No amplicons were observed with any of these

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Table 6. Analysis of *Xanthomonas arboricola* pv. *pruni* in cherry suspected samples by isolation, optimised conventional PCR and real-time PCR.

Cherry cultivar	Organ	Samples (No.)	Orchards or origins (No.)	Isolation	Conventional PCR	Conventional PCR 1:10	Real-time PCR
nd	Leaf	2	1	-	-	-	+
nd	Leaf	8	2	-	-	-	-
nd	Stem	2	1	-	-	-	-
Ambrunes	Leaf	6	1	-	-	nd	-
Prime Giant	Leaf	3	1	-	-	nd	-
Prime Giant	Canker	3	1	-	-	nd	-
A7-91C	Leaf	10	1	-	-	nd	-
Prime Year	Canker	10	1	-	-	nd	-

+, positive; -, negative; nd, not determined

44 strains when assayed with the modified protocol (data not shown). However, non-desired reactions were obtained with strains of *X. arboricola* pv. *corylina*, a quarantine pathogen of hazelnut not reported from *Prunus* spp. (data not shown), according to results also obtained by Pothier *et al.* (2011a).

Related to the sensitivity of the modified protocol, it was able to detect *Xap* in almond leaves spiked with 10^2-10^3 CFU ml^{-1}, in apricot leaves spiked with 10^5 CFU ml^{-1}, in Japanese plum leaves spiked with 10^5-10^6 CFU ml^{-1}, in peach leaves spiked with 10^2 CFU ml^{-1} and in peach x almond hybrid GF-677 leaves spiked with 10^5-10^6 CFU ml^{-1} (Table 1). Surprisingly, the sensitivity limits observed with the modified PCR protocol for *Xap* detection in spiked samples of apricot was quite poor, *ca* 1,000-fold less than with suspensions of *Xap* cultures, and also for Japanese plum and GF-677, even 10,000-fold less. These results were probably caused by the amount of inhibitors of these plant extracts, such as phenolics, thus suggesting that the DNA extraction method utilised was not efficient enough. Consequently, the dilution 1:10 of the extracts was advised in order to reduce the inhibitors concentration, to improve sensitivity and minimize the risk of false negative results. Inhibition was also reported in real-time PCR analysis, particularly in plum samples (Palacio-Bielsa *et al.*, 2011). The inhibitors content can be variable not only

Table 7. Comparative analyses of cherry field samples and percentage of congruence isolation-optimised conventional PCR, isolation-real-time PCR and real-time PCR-optimised conventional PCR.

		Conventional PCR	
		+	-
Isolation	+	0	0
	-	0	44

+, positive; -, negative
Congruence isolation-optimised conventional PCR: 100%

		Real-time PCR	
		+	-
Isolation	+	0	0
	-	2	42

+, positive; -, negative
Congruence isolation-real-time PCR: 95.46%

		Conventional PCR	
		+	-
Real-time PCR	+	0	2
	-	0	42

+, positive; -, negative
Congruence real time-PCR-optimised conventional PCR: 95.46%

Table 8. Analysis of *Xanthomonas arboricola* pv. *pruni* in Japanese plum suspected samples by isolation, optimised conventional PCR and real-time PCR.

Japanese plum cultivar	Organ	Samples (No.)	Orchards or origins (No.)	Isolation	Conventional PCR	Conventional PCR 1:10	Real-time PCR
nd	Leaf	10	1	-	-	nd	-
nd	Leaf	7	3	-	-	-	-
nd	Leaf	1	1	+	-	+	+
nd	Leaf	1	1	-	-	-	+
nd	Leaf	4	2	+	-	-	+
nd	Leaf	1	1	+	-	-	-
nd	Fruit	10	1	-	-	nd	-
nd	Fruit	10	1	-	-	-	-
nd	Fruit	9	1	+	+	+	+
nd	Fruit	1	1	-	+	-	+

+, positive; -, negative; nd, not determined

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Table 9. Comparative analyses of Japanese plum field samples and percentage of congruence isolation-optimised conventional PCR, isolation-real-time PCR and real-time PCR-optimised conventional PCR.

		Conventional PCR	
		+	-
Isolation	+	10	5
	-	1	38

+, positive; -, negative
Congruence isolation-optimised conventional PCR: 88.89%

		Real-time PCR	
		+	-
Isolation	+	14	1
	-	2	37

+, positive; -, negative
Congruence isolation-real-time PCR: 94.44%

		Conventional PCR	
		+	-
Real-time PCR	+	11	5
	-	0	38

+, positive; -, negative
Congruence real time-PCR-optimised conventional PCR: 90.74%

in different cultivars of the same species but also because some chemicals, such as copper, used in crop treatments may act as PCR inhibitors (Pulawska *et al.*, 1997; López *et al.*, 2003). This could explain the very different sensitivity level in apricot obtained by Pothier *et al.* (2011a) compared to our results. Several DNA extraction kits such as DNeasy (Qiagen) were also tested without improving the sensitivity (data not shown).

Data of comparative analyses of field samples from five different hosts are detailed (Tables 2, 4, 6, 8 and 10) as well as the congruence percentage of isolation-conventional PCR, isolation-real-time PCR and real-time PCR-conventional PCR (Tables 3, 5, 7, 9 and 11). It was concluded that in some hosts, the observed disorders were not caused by *Xap* in most cases, thus confirming the possible confusion of bacterial spot disease with other biotic or abiotic causes of lesions found in Spain.

From 44 of the 127 *Xap*-suspected almond samples the target bacterium was recovered but only 38 of them were positive by conventional PCR, possibly because in the other six cases the bacterial populations were below the detection limit of the technique. Forty three samples were positive in real-time PCR (Table 2). So, in almond samples, isolation and conventional PCR were congruent in 95.28% of the samples, isolation and real-time PCR in 99.21% of the samples and real-time PCR and conventional PCR in 96.06% (Table 3).

From the 30 *Xap*-suspected apricot samples, the target bacterium was culturable only from 12 samples, 8 of which were also positive by conventional PCR (Table 4). Ten samples were positive by real-time PCR. Thus, in the analyzed samples, isolation and conventional PCR were congruent in 86.67% of the samples, isolation and real-time PCR in 93.33% of the samples and real-time PCR and conventional PCR in 93.33% (Table 5).

Xap was never isolated from the 44 *Xap*-suspected cherry samples and only two were positive by real-time PCR (Table 6). In this case, isolation and conventional PCR were congruent in 100% of the samples, isolation and real-time PCR in 95.46% of the samples and real-time PCR and conventional PCR in 95.46% of the cases (Table 7).

From the 54 *Xap*-suspected Japanese plum samples, the bacterium was isolated only in 15 cases. By conventional PCR, 11 samples were positive and 16 were posi-

Table 10. Analysis of *Xanthomonas arboricola* pv. *pruni* in peach suspected samples by isolation, optimised conventional PCR and real-time PCR.

Peach cultivar	Organ	Samples (No.)	Orchards or origins (No.)	Isolation	Conventional PCR	Conventional PCR 1:10	Real-time PCR
nd	Leaf	15	4	-	-	-	-
nd	Leaf	9	3	-	-	-	+
nd	Leaf	7	3	+	-	+	+
nd	Leaf	1	1	+	-	-	-
nd	Leaf	1	1	+	-	-	+
nd	Leaf	2	2	+	+	+	+
nd	Shoot	2	1	-	-	-	-
nd	Stem	1	1	-	-	-	-
nd	Bud	1	1	-	+	+	+
nd	Bud	1	1	-	-	-	-
nd	Bud	1	1	-	-	-	+
Baby Gold	Stem	1	1	-	-	nd	-
Baby Gold	Canker	9	1	-	-	nd	-
Flor de Star	Fruit	10	1	-	-	nd	-

+, positive; -, negative; nd, not determined

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Table 11. Comparative analyses of peach field samples and percentage of congruence isolation-optimised conventional PCR, isolation-real-time PCR and real-time PCR-optimised conventional PCR.

		Conventional PCR	
		+	-
Isolation	+	9	2
	-	1	49

+, positive; -, negative
Congruence isolation-optimised conventional PCR: 95.08%

		Real-time PCR	
		+	-
Isolation	+	10	1
	-	11	39

+, positive; -, negative
Congruence isolation-real-time PCR: 80.33%

		Conventional PCR	
		+	-
Real-time PCR	+	10	11
	-	0	40

+, positive; -, negative
Congruence real time-PCR-optimised conventional PCR: 81.97%

tive by real-time PCR (Table 8). The percentage of congruence was 88.89% between isolation and conventional PCR, 94.44% between isolation and real-time PCR and 90.74% between real-time PCR and conventional PCR (Table 9).

Only from 11 of the 61 *Xap*-suspected peach samples, the target bacterium was isolated. Ten samples were positive by conventional PCR and 21 positive by real-time PCR (Table 10). In the analyzed samples, isolation and conventional PCR were congruent in 95.08% of the samples, isolation and real-time PCR in 80.03% of the samples and real-time PCR and conventional PCR in 81.97% of the samples (Table 11).

These results suggest that the lesions in many *Xap* suspected samples, could be due to other causes, because *Xap* was not found in many of the samples analysed regardless of the technique used. This was especially significant in two hosts, in which most of the samples (i.e cherry), or a high proportion of them (i.e. almond) were negative. However, the recent detections of *Xap* in some Spanish regions (Palacio-Bielsa *et al.*, 2010a,b; Roselló *et al.*, 2010; Roselló *et al.*, this issue) demonstrate the accuracy of the visual recognition of *Xap*-specific symptoms in some areas of Spain.

The sensitivity of the isolation method employed was quite good with the suspected or symptomatic samples analyzed. Only in a low number of samples (22 out of 316), isolation was positive whereas conventional PCR (17 out of 316) or real-time PCR (5 out of 316) were negative, showing that these techniques are suitable for rapid screening. It was observed that when bacterial population levels recovered on plates were low (1-10^2 CFU ml^{-1}), also conventional PCR (or even real-time PCR) was often negative, either because the bacterial populations were below the detection threshold of both techniques and/or because of inhibition by compounds was still present after DNA extraction.

Some samples were positive by molecular techniques but no colonies of the target bacterium were isolated from them. This only happened in relatively few cases (i.e. one Japanese plum and one peach sample positive by conventional PCR, and two cherry, two Japanese plum and eleven peach samples positive by real-time PCR). Special difficulties on isolating *Xap* from plant material might explain these cases, since the companion microbiota can interfere with the growth or mask *Xap* colonies. Another possibility is that PCR may amplify DNA from dead *Xap* cells or DNA from other bacteria present in plant extracts.

Related to the other conventional PCR protocols currently available, the information about their efficiency in different hosts and organs is still quite limited. The PCR protocol described by Park *et al.* (2010) was only evaluated with peach samples (no other *Prunus* species were tested), detecting successfully *Xap* from symptomatic fruits. The duplex-PCR described by Pothier *et al.* (2011a) was also used exclusively with symptomatic peach and apricot samples naturally infected by *Xap* and no comparison was made between PCR and isolation. The multiplex-PCR described by Pothier *et al.* (2011b) has not yet been tested with plant material and the real-time SYBR Green I Bio-PCR assay developed by Ballard *et al.* (2011) has only targeted naturally infected plum samples.

The results of our analyses have shown that a high level of congruence exists between the positive and negative results obtained by the three mentioned techniques in the five *Xap* hosts tested. These data, and those relative to the specificity and sensitivity of the protocol, make this optimised conventional PCR an excellent candidate for the analysis of symptomatic samples from different types of organs and *Xap* hosts, when a rapid result is required and real-time PCR equipment is not available.

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